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Investigation of mesenchymal stem cells and the development of experimental strategies for rescuing glaucomatous eyes using a stem cell-based therapy

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Investigation of mesenchymal stem cells and the development of experimental strategies for rescuing glaucomatous eyes using a stem cell-based therapy

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TABLE OF CONTENTS

ACKNOWLEDGMENTS……..……..vii

ABSTRACT ..... ……..…… viii

CHAPTER 1: GENERAL INTRODUCTION ……...……... 1

Introduction …….……..…….. 1

Dissertation Organization …….….. .2

Literature Review ……..3

Overview …….…….....3

The Mammalian retina ……..…..…… 4

Glaucoma …….…… 7

Animal models of glaucoma ...……..…….....8

Genetic model of Glaucoma_DBA/2J mouse ……..……..10

Additional genetic Model of Glaucoma_Myocilin (MYOC)-mutant mouse……..…...……..…12

Neurotrophic factors ……..……..14

Brain derived-neurotrophic factor (BDNF) and Retina ……..……..15

Glial cell-derived neurotrophic factor (GDNF) and Retina …….....16

Neurotrophic factors and Treatment of Retinal Damage ……..…..…...17

Mesenchymal stem cells ……..……...19

Cellular-based retinal therapies …….……..……...……..…21

Viral constructs ……..……..……..…...22

Retinal functional measurement ……..……..……...23

Summary ……..…..…25

References ……...…..……..…...26

CHAPTER 2: BONE MARROW-DERIVED MESENCHYMAL STEM CELLS STIMULATE NEURITE OUTGROWTH FROM ADULT HIPPOCAMPAL PROGENITOR CELLS……..…..….42

Abstract ……42

Introduction ……..…..44
Materials and Methods ……..…..45
Animals ……..……..……45
MSC isolation and culturing ……46
Population doubling time ……..…….…..…47
5-bromo-2-deoxyuridine (BrdU) assay ……..…..…...……47
Propidium iodide (PI) staining ……..……..…..…48
Adipogenesis ……..……..…..…48
Osteogenesis ……..……..…..…49
Co-Culture of MSCs with AHPCs ……..……..…49
Immunocytochemistry ……..……..…50
Quantification of neurite outgrowth ……..……..…...51
Imaging and statistics ……..…..51
Results ……...…...52
Isolation and characterization of Brown Norway rat MSCs ……..……52
MSC growth rate and proliferation ……...……53
Cell Viability ……..……..…..54
Differentiation of MSCs into mesodermal lineages ……..…..……...54
Co-culture of MSCs with AHPCs ……..……..…..…55
Discussion ……..…….....57
Acknowledgments ……..……..…….....63
References..……..……..……..……...63
Figure Legends……..……....….68
Tables and Figures ……..……..……...74

CHAPTER 3: ENGINEERING MOUSE MESENCHYMAL STEM CELLS DERIVED FROM BONE MARROW AND *IN VITRO* CHARACTERIZATION ……....83
Abstract ……...……...……..83
Introduction ……..……..……....……...84

Materials and Methods ……...……..86
Mouse mesenchymal stem cells …….……...86
Engineering mesenchymal stem cells Ex-Vivo with lentiviral vectors….87
ELISA of BDNF and GDNF production …….……88
Bioactivity assay of neurotrophic factors …….……....89
Immunocytochemistry …….…….……....90
Quantitative analysis of neurotrophic factor expression on the MSCs…..91
Imaging and statistics ..…….……..……..91
Results ……...……92
Increased expression of neurotrophic factors in the MSCs. …….……92
Enhanced secretion of neurotrophic factors from the MSCs……..…...…93
Bioactivity of neurotrophic factors released from the MSCs……...…..…94
Discussion …….……95
Acknowledgments …….……...……....98
References …...……..……98
Figure Legends……104
Tables and Figures…….……...……...107

CHAPTER 4: FUNCTIONAL AND HISTOLOGICAL EVALUATIONS OF THE RETINA AND OPTIC NERVE OF DBA/2J MICE AFTER MSC TRANSPLANTATION……...115

Abstract ……..115
Introduction ..……..117
Materials and Methods ……...120
Animals ……...…….……...120
Mouse mesenchymal stem cells (MSCs) ..……...…….121
Engineering MSCs *Ex Vivo* with lentiviral vectors……...……..…121
Cell transplantation …….……122
Measurement of intraocular pressure (IOP) ……123

Pattern electroretinogram (PERG) ……..…123
Tissue preparation and immunohistochemistry ……..……..……...124
Quantification of retinal layer thickness ……..……...……125
Retinal imaging and statistics ……...……..126
Results ……...126
IOP measurement after MSC transplantation ……..…….…….……126
Assessment of functional RGCs ……..……..127
Survival of transplanted MSCs in adult DBA/2J mice ……..……128
Qualitative analysis of retinal structure of the host retinas after MSC transplantation..……...…….....128
Assessment of retinal layer thickness …….……...129
Changes of RGC and acetyl-cholinesterase (ChAT)-IR amacrines in the host retina.…….....….…….....130
Discussion ……..…….……...130
References …..……...…….....135
Figure Legends……..…..……..138
Tables and Figures…….….……142

CHAPTER 5: HISTOLOGICAL EVALUATIONS OF RETINAS FROM MYOCILIN (MYOC)-MUTANT MICE AFTER MSC TRANSPLANTATION……..152

Abstract ……..……152
Introduction ..……..……153
Materials and Methods …….…….……..…155
Animals …….……..……155
Mouse mesenchymal stem cells (MSCs) …….....156
Engineering MSCs *Ex Vivo* with lentiviral vectors ……..……..…....156
Cell transplantation ……...……..…157
Tissue preparation and immunohistochemistry ……...……...158
RGC counting ……...……..…159

Imaging and statistics ……..…159
Results ……...…….……160
Long-term survival of MSCs in adult eyes of MYOC-mice ……...…...160
Assessment of RGC survival after MSC transplantation ……...……161
Expression of myocilin on the ocular tissues ……...……..…161
Discussion ……..……162
References ……..……...……..…..….164
Figure Legends……...…….……..167
Tables and Figures…….……..…….…….....169

CHAPTER 6: GENERAL CONCLUSIONS …….……..……..…174

RECOMMENDATIONS FOR FUTURE RESEARCH ……...……..……176

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ABSTRACT

Glaucoma, the second leading cause of blindness in the world, is a hereditary ocular disease and about 60 million people suffer from it [1]. However, currently there are no remedies to cure glaucoma. As a potential solution to rescue degenerating retinas, stem cells have been investigated extensively and the effects of morphological neuroprotection or functional recovery have been reported [2-5]. In the present studies, I aimed to investigate two main topics of research. From the first topic, we demonstrated MSCs from Brown Norway rats are a self-renewing, multi-potent population. More importantly, we provide the first evidence that bone marrow MSCs are capable of promoting neurite outgrowth from adult hippocampal progenitor cells (AHPCs).

For the second topic of research, mouse MSCs were engineered to over-express brain-derived neurotrophic factor (BDNF) and/or glial cell-derived neurotrophic factor (GDNF) together with green fluorescent protein (GFP), as a strategy for stem cell-based therapy. Our results show MSCs, infected with lentiviral vectors encoding BDNF or GDNF, noticeably increased the release of bioactive neurotrophic factors *in vitro*.

For our *in vivo* study of stem cell-therapy, two different mouse models of experimental glaucoma were studied. MSCs, following intraocular transplantation, survived up to 4 months post-transplant (PT) in the host retina of adult DBA/2J mice. Interestingly, our results indicated MSCs expressing GDNF played a potential role in alleviating glial activation on host retinas. Importantly, our data show GDNF/BDNF-MSCs contributed to preservation of the inner retina of the recipient eye, compared to the control fellow eye.

An additional transgenic mouse model of glaucoma, Myocilin (MYOC)-mutant mice, was investigated. Our preliminary study showed transplanted MSCs survived up to 11.5 months-PT in the adult host retina. No significant changes in the number of retinal ganglion cells between experimental and control eyes were found at 11.5 months-PT.

These studies are the first to characterize bone marrow-derived MSCs from Brown Norway rats and to study potential neuroprotective effects of transplanted MSCs on the host retinas of DBA/2J and MYOC-mice. This study provides an experimental strategy towards clinical applications in terms of using MSCs as a potential autograft and long-term neuroprotection via neurotrophic factor delivery for retinodegenerative diseases.

CHAPTER 1: GENERAL INTRODUCTION

Introduction

Mesenchymal stem cells (MSC) are a potential resource for stem cell therapy, and a very attractive, promising candidate for syngeneic transplant. Glaucoma, the second leading cause of blindness in the U.S., is a hereditary optic neuropathy. There is no remedy to cure the disease, yet. Many studies have been performed to develop therapeutic strategies of neurodegenerative ocular diseases. However, there still exist great needs for investigation to establish robust resources of stem cells and to develop long-term strategies of stem cell therapy. In these series of experiments, I investigated a new line of mesenchymal stem cells and therapeutic strategies for stem cell-based therapy on transgenic mouse models of experimental glaucoma.

The hypotheses for this work are: 1) mesenchymal stem cells isolated from the bone marrow of Brown Norway rats are self-renewing, multipotent, and capable of stimulating neurite outgrowth from neural stem/progenitor cells; and 2) mesenchymal stem cells can be engineered to over-express bioactive neurotrophic factors and serve as a potential transplant for models of experimental glaucoma.

A set of specific aims was generated to test the hypotheses.

Specific Aim I: Isolate and characterize mesenchymal stem cells from the bone marrow of Brown Norway rats and investigate the capability of stimulating neurite outgrowth from adult hippocampal progenitor cells (AHPCs).

Specific Aim II: Engineer mouse mesenchymal stem cells derived from bone marrow and perform i*n vitro* characterization.

Specific Aim III: Perform functional and histological evaluations of the host retina and optic nerve of glaucomatous eyes after MSC transplantation.

Dissertation Organization

This dissertation is organized in the alternative format, which includes two manuscripts prepared for submission. The manuscripts are organized into three separate research chapters.

1. **Eun-Ah Ye**, Sagar Chawla, Michael Khan, and Donald S. Sakaguchi. Bone marrow-derived mesenchymal stem cells stimulate neurite outgrowth from adult hippocampal progenitor cells. Prepared for submission to Journal of Neuroscience Research.

2. Eun-Ah Ye, Jacob Rysted, and Donald S. Sakaguchi. Long-term strategy of rescuing glaucomatous eyes: Transplant of bone marrow-derived mesenchymal stem cells engineered for production of neurotrophic factors into the eyes of DBA/2J mice. Prepared for submission to Stem Cell Research.

In addition to the three research chapters, the fourth research chapter presents a preliminary study of an additional glaucoma model, the Myocilin transgenic mouse model. The final chapter of this dissertation contains general conclusions and recommendations for future research with regards to these projects.

Literature Review

Overview

The central nervous system (CNS), including the retina, plays a pivotal role in the body. However, the mammalian CNS lacks regenerative abilities. Therefore, the development of therapeutic strategies for neurodegeneration is of great interest. Currently, millions of people in the U.S. suffer from ocular diseases, which eventually can lead to vision loss. Glaucoma, the second leading cause of blindness in the U.S., is a hereditary ocular disease and about four million Americans are affected. At the moment, no remedies to cure glaucoma exist and most treatments focus on lowering elevated intraocular pressure (IOP). These approaches do not provide neuroprotection on the degenerative eye. Stem cell therapy, as a potential treatment for neurodegenerative disorders, has been investigated extensively and much evidence showing significant neuroprotection has been reported. Establishing reliable and robust resources for stem cell lines are important to provide broader accessibility of the usage for research and clinical therapy. Moreover, developing long-term strategies of stem cell-based therapy may provide a potent solution to cure challenging, disastrous neurological diseases. The results presented within this dissertation focus on 1) characterizing a new cell line of rat MSCs as a potential resource for transplantation studies in rats and 2) developing long-term strategies of neuroprotection on glaucomatous eyes of transgenic mouse models. The resource for this new rat MSC line will be useful not only for the development of treatment strategies of glaucomatous eyes, but also for a variety of other neurodegenerative conditions. In addition, therapeutic strategies proposed here show potential for long-term treatment on glaucoma via the engineered MSCs possible with autotransplants. Thus, this study has significance in the clinical setting.

The Mammalian retina

The retina is a light-sensitive tissue lining the back of the eye, 200 µm thickness in rodents [6], and is a part of the central nervous system. The retina is the first region visual processes occur and sends the integrated information to the targeted areas of the brain via the optic nerve.

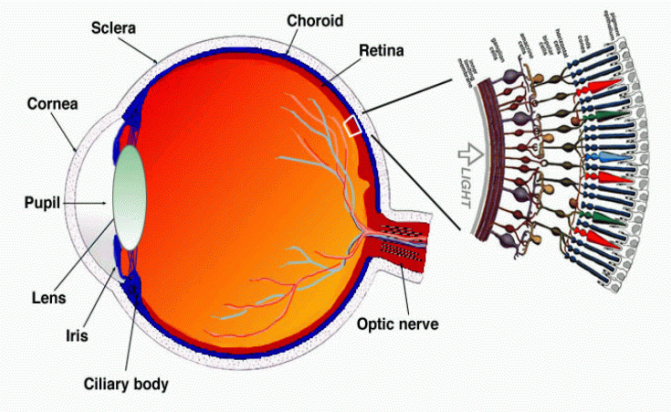
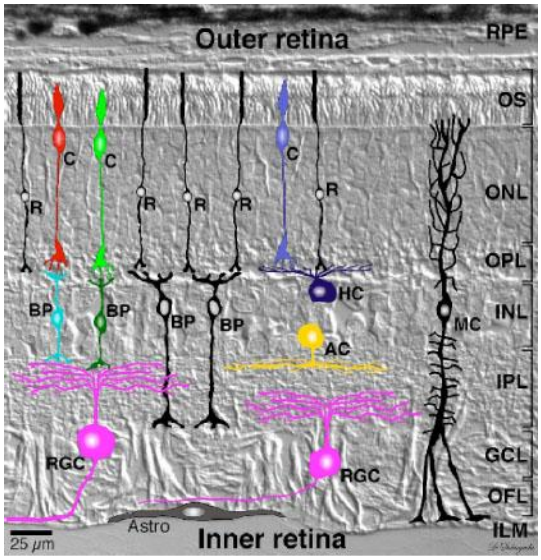


Figure 1. Structure of adult human eye. (Citation-http://webvision.med.utah.edu/)



The retina is an excellent model of CNS development, due to its relatively simple structure. The retina is developed from the anterior neural tube. The anterior neural tube develops to form optic vesicle and, subsequently, optic cup. The neural retina is formed from the inner layer of the optic cup [7, 8].

Figure 2. Structure of adult human retina. Image from Dr. Donald Sakaguchi; Abbreviations- RPE: retinal pigment epithelium, OS: outer segments, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer, OFL: optic nerve fiber layer, ILM: inner limiting membrane, C: cones, R: rods, HC: horizontal cells, BP: bipolar cells, AC: amacrine cells, RGC: retinal ganglion cells.

All vertebrate retinas have the same structural basis, consisting of seven major classes of cells and two major synaptic layers [7]. The developmental processes are highly regulated by

intrinsic and extrinsic cues, and vertebrate retina generally follows the same pattern of retinogenesis with some variations between species [7]. Retinal ganglion cells are generated first, followed by cones, amacrine cells, and horizontal cells. Then, rod photoreceptors are formed, followed by bipolar cells, and finally, Müller glia. An overlap in the time points of cell birth exists during development. Development of the retina in the mouse begins at embryonic day 10 (E10) and is fully developed a few days after birth [7, 8].

A fully developed retina is a complex neural circuit. All vertebrate retinas consist of three nuclear layers and two plexiform layers. Six different classes of neurons make up the retina—rods, cones, horizontal cells, bipolar cells, amacrine cells, and retinal ganglion cells. In addition, three types of glial cells exist in the retina—Müller glia, astrocytes, and microglia [9]. The rod and cone photoreceptors, comprising the outer nuclear layer, are the largest populations in the retina and initiate the process of phototransduction. Rod photoreceptors outnumber cones by twenty fold in most mammals [10]. The outer plexiform layer, the location of the first synapses in the retina, processes the visual information from the rods and cones to the second order neurons. Various bipolar and horizontal cells, located in the inner nuclear layer, transmit the information from the photoreceptors to the inner plexiform layer. The inner plexiform layer is the synaptic region where bipolar, amacrine, and retinal ganglion cells interact. Retinal ganglion cells, the final output neurons, receive the visual message from the two nuclear layers and send the information to the target areas of the brain via optic nerves.

There are subtypes of each major class of retinal neurons. Mammalian retinas have about 55 different specific cell types. Retinal cell types are classified based on dendritic morphology, the sizes of cell body and dendritic tree, or cell stratification. Two main types of

photoreceptor, cones and rods, exist. There are 9–11 types of cone-bipolar cells and a type of rod-bipolar cell, two types of horizontal cells, 29 types of amacrines, and 10-15 types of retinal ganglion cells in a typical mammalian retina [10].

In addition to the neuronal populations, there are three major classes of glial cells in the retina. Müller glia, as the principal type of intrinsic macroglia in the retina, provides structural support spanning the thickness of the retina, and forming the outer and inner limiting membranes. Müller cells elicit cellular depolarization as they express many neurotransmitter receptors and voltage-gated channels. Also, they regulate neuronal activity by controlling the concentrations of ions and neurotransmitters in the extracellular space [11]. Müller cells work as an accelerator for the process of neuronal degeneration, or mediate the protection of nerve cells by secreting trophic factors and uptaking excitotoxin glutamate [12]. Very interestingly, they play a crucial role in vision by serving as a low-scattering passage for light from the innermost layer to the photoreceptors [13].

The second type of macroglia in the retina are the astrocytes. Astrocytes are primarily restricted to the nerve fibre layer and they distribute only in vascularized region. The astrocytes migrate from the brain along the optic nerves [14]. Gap junctional coupling between retinal astrocytes, between Müller cells, and between astrocytes and Müller cells has been reported [15]. Astroglial activities, such as hypertrophy and phagocytosis of dead RGCs, are involved in the pathological conditions of retina [16] .

The third type of glia of the retina are the microglia. These cells are distributed broadly in the inner part of retina, mostly locating in the inner plexiform layer and nerve fiber layer [17]. Microglia are cells of hemopoietic origin and invade the retina via blood

vessels of the optic disc, iris, and ciliary body. Microglia function in host defense against foreign microorganisms, and they invade and become activated under pathological conditions in the retina [18-20]. It is likely that microglial cells contribute to the regenerative processes [21, 22]

Glaucoma

Glaucoma is a group of ocular diseases and multi-factorial disorders [23]. In the U.S., millions of people suffer from ocular diseases, which eventually can lead to vision loss. There are 60 million people affected in the world and 8.4 million among them are blind bilaterally [1]. Glaucoma is the second leading cause of blindness in the world, and is asymptomatic until visual problems occur. It is known that genetic background, as well as environmental factors, influences the development of glaucoma. One of the significant factors that contribute to glaucoma is elevated intraocular pressure (IOP). Death of retinal ganglion cells (RGCs) is the most significant result, causing vision loss.

There are two major types of glaucoma from which most subjects suffer: (1) open-angle glaucoma and (2) angle-closure glaucoma [1]. Open-angle glaucoma is characterized by the fact that intraocular pressure of the subject is often in the normal range, but associated factors, such as stress related to the pressure, contribute to glaucoma. This type of glaucoma progresses slowly and is usually asymmetric and bilateral. Genetic background is also influential. The ethnic origin of Africans shows a relatively high prevalence of glaucoma [24-26].

Angle-closure glaucoma is categorized by the symptoms of elevated intraocular

pressure resulting from obstruction of the outflow of aqueous humor, due to the angle closure between the iris and trabecular meshwork. This glaucoma shows progressive and asymmetric loss of vision like open-angle glaucoma, but a sudden decrease of visual ability occurs. This type of glaucoma is found in one-third of the subjects with primary glaucoma worldwide. About twice the number of people with angle-closure glaucoma show vision loss compared to those with open-angle glaucoma. Genetic background is also influential in angle-closure glaucoma and a known risk factor is small size of the eye. It is known that the ethnic origin of Indians and Asians, women, and older individuals have higher risks of developing angle-closure glaucoma [27].

The pathogenesis of glaucoma includes characteristic phenomena. The connective tissues at the optic disc are altered and that induces the excavation of the optic cup. This occurs coincidently with the early axonal abnormalities [28]. Damaging effects at the optic nerve head result in axon injury, and both retrograde and anterograde axonal transport are blocked [29]. Primary degeneration of RGCs appears to cause secondary RGC death with a harmful environment, resulting from the generation of free radicals [30], glutamate excitotoxicity [31], and impaired functioning of immune responses [32]. There are common pathways for neuronal degeneration in Alzheimenr's disease and glaucoma, such as deposition of amyloid-beta [33] and decrease of nerve fiber layer (NFL) thickness [34].

Animal models of glaucoma

Research into neurodegenerative diseases, including glaucoma, is quite extensive. Yet, the molecular and physiological mechanisms underlying glaucoma are not completely

understood. *In vivo* studies are essential to reveal the complicated phenomena occurring in the organisms. Animal models for glaucoma are required to understand why and how the death of retinal cells occur, and to develop new strategies to treat the disease. Currently, there are no complete animal models that mimic human glaucoma. Thus, developing a model is very challenging and complex work. So far, several models in different species have been developed in order to study experimental glaucoma [35]. The monkey has a high homology with humans and the anatomy of the eye is almost the same as in human eyes, but monkeys are difficult to handle, expensive, and there is limited availability. Three different methods have been developed using the monkey. Argon laser photocoagulation [36] and diode laser photocoagulation [37] induced a moderate increase of intraocular pressure (IOP) and optic nerve cupping. Another method, injection of autologous blood cells into the anterior chambers resulted in extended IOP elevation [38, 39]. However, it reduced the ability to visualize the retina and optic nerve head. Additionally, injection of latex microspheres into the anterior chamber induced different durations and levels of IOP elevation. This method does not require expensive equipment and does not block the visual field to examine the optic disc [40].

Mice and rats are common models to study ocular diseases and have been extensively used. They are easy to handle, relatively inexpensive, and their genome has considerable conservation with humans. Also, their lifespan is about two years and they develop diseases relatively in a short time frame, compared to humans, who take decades to develop. Mice provide even greater availability than rats, since there is a variety of knockout and transgenic mice [41] which greatly facilitates investigations into the genes and pathology involved in the diseases.

In rats, several methods to elevate IOP are currently available [35]. Translimbal laser photocoagulation is simple, reproducible, reliable, and produces a sustained elevation of IOP [42]; whereas, other methods available are more difficult and time consuming. Cauterization of large veins [43, 44], injection of hyperosmotic saline into the episcleral veins [45, 46], and injection of India ink into the anterior chamber a week prior to laser photocoagulation [47], all induce elevated IOP in the rats. All four of these methods produce acute models of experimental glaucoma and sometimes require repetition of the procedure, if the IOP difference between experimental and control eyes is not significantly large. In addition to these models, magnetic microspheres were used to induce a sustained IOP elevation by injecting into the anterior chamber. This method is simple and effective to induce experimental glaucoma [48]. Also, polystyrene microbeads injected into the anterior chamber caused ocular hypertension [49].

In mice, both laser photocoagulation and injection of hyperosmotic saline on the limbus [35], and polystyrene microbeads [49] work successfully to induce IOP elevation and axonal loss. In addition to these acute models of glaucoma, genetic models are also available in mice. These are transgenic mice and more suitable for the study of chronic glaucoma compared to the acute models. Two major models of transgenic mice are currently available: (1) DBA/2J and (2) myocilin-mutant (MYOC) mice. Details about these models are discussed in the following sections.

Genetic model of glaucoma_DBA/2J mouse

The DBA/2J mouse is a hereditary and spontaneous glaucoma model. It is a very good tool to study experimental glaucoma, because the mouse model is well-investigated and

has characteristics of human glaucoma, including age-related progression of IOP elevation and RGC death, and excavation of the optic nerve head [50].

DBA/2J mouse is an inbred mouse strain and homozygous for both *Tyrp1*^{b} and *Gpnmb*^{R150X} mutations [51]. These mutations generate iris diseases in the mice, which are iris stromal atrophy and iris pigment dispersion [52]. Iris stromal atrophy is caused by a mutant allele of tyrosinase related protein 1 (*Tyrp1*^{b}) [51] and has the characteristics of deteriorating iris stroma and the blockage of the ocular drainage with stromal cell fragment and pigment [52]. Iris pigment dispersion is caused by a mutation in *Gpnmb*^{R150X} gene and is characterized by pigment dispersion and transillumination defects [51, 53]. Two other strains of DBA/2J, D2.*Tyrp1B6GpnmbB6* and D2-*Gpnmb*+, were generated by replacing the mutant alleles of either both *Tyrp1 and Gpnmb* or only *Gpnmb* by wild-type allele. None of the strains showed elevated IOP or glaucoma [54].

Different aspects of glaucomatous phenomena have been investigated on the mouse model. DBA/2J mice have shown a linear increase of IOP from 2 to 10 months of age- from 15~16 to 22~23 mmHg. IOP is correlated with the preservation of the optic nerve, as well as corneal thickness [55]. It was also reported that DBA/2J mice showed a progressive decrease of pattern electroretinogram (PERG) amplitude after 3 months of age and the amplitude reached the noise level by 11 months [56]. In addition, visual performance of DBA/2J mice has been studied behaviorally using different types of methods.

DBA/2J mice showed decreased visual performance in the visual water task starting around 7 months of age. Optomotor test also showed that the visual acuity of aging DBA/2J mice was significantly decreased about 7 months of age.[57].

In addition, many glaucomatous characteristics of morphological changes in the eyes

of DBA/2J mice have been reported. DBA/2J mice show timeline changes of RGCs. The deficits in axonal transport, found after age 6 months, precedes axonal loss and cell loss (NeuN-IR), observed at about 13- and 18-months old mice, respectively [58]. Another study reported that axonal loss and gliosis in the optic nerve are evident in DBA/2J mice around 8 to 9 months of age. Loss of RGCs, examined by âGeo expression, is apparent about 8 to 10 months of age and is predominant after 10.5 months of age [59]. A study on DBA2/NNia mice reported preferential cell loss of larger RGCs [60], and the other study showed topological RGC loss, not cell type-specific, in DBA/2J mice [61].

In addition to the degeneration of RGCs, glial changes in the retina and optic nerve are also crucial factors involved in the progression of glaucoma. Increased reactivity on the retinal astrocytes and Müller cells, and increased number of microglia were shown in aging DBA/2J mice after 3- to 4-months of age [62, 63]. Also, in the optic nerves of aged DBA/2J, the loss of oligodendrocytes, proliferation of oligodendrocyte precursor cells, new oligodendrocyte generation, an early increase in astrocyte reactivity, and activation of microglia were shown [64]. Moreover, correlations between (1) corneal thickness with IOP [55], (2) the number of remaining oligodendrocytes and the number of axons, and (3) the activation of resident microglia to axon degeneration in the optic nerves [64] have been reported.

Additional genetic model of glaucoma_Myocilin (MYOC)-mutant mouse

MYOC-mouse, in addition to the DBA/2J mouse, is an additional chronic model of experimental glaucoma. In humans, there is an inheritance factor in primary open-angle

glaucoma (POAG) and mutations in MYOC are found in 3-4% of people with POAG [65], and more than 70 *MYOC* mutations have been revealed [66]. Glaucoma associated with Myocilin appears to be inherited with autosomal dominant pattern [67, 68]. Mutations in MYOC have been referred to as "glaucoma-causing mutations" as they are able to cause the development of POAG on their own without influence from the environment or other genes. In contrast, glaucoma risk alleles, are another group of mutations and they would cause POAG when associated with environment or other alleles [69]. In addition, there are differences of ethnicity on MYOC mutations. One of the most common mutations, GLN368STOP, has been found in almost all POAG populations including Caucasians and African-Americans. However, the mutation has not been detected in Asian patients [65].

Myocilin contains 504 amino acids [70] and has a molecular weight of about 55–57 kDa [71]. Initially, it was found that Myocilin is distributed preferentially in the connecting cilium of photoreceptors and suggested as a novel cytoskeletal protein playing a role in the morphogenesis of photoreceptors [71]. This protein is a secreted glycoprotein that is highly expressed in the trabecular meshwork, ciliary body, sclera, and iris, and also expressed in retina and optic nerve head at low amounts [72]. More specifically, myocilin was localized in the cells of iris stroma, ciliary epithelium, and smooth muscle cells forming the blood vessels and iris muscles in the anterior part of the eye [73]. In the trabecular meshwork, cells in the corneoscleral and uveal meshwork and also extracellular matrix of the juxtacanalicular region express myocilin. These regions are located nearby Schlemm's canal [74]. It has been suggested that myocilin plays a role in the interaction of cell and matrix, but the functions of myocilin are not fully understood, yet [65, 72, 75, 76].

MYOC-mice, Tg-MYOC^{Y437H}, were generated by using a human MYOC gene modified to introduce a point mutation. These mice express human *MYOC, which contains the* mutation under the control of the CMV promoter, within ocular tissues including trabecular meshwork [77]. It has been reported mutant Myocilin accumulates in the endoplasmic reticulum (ER), and results in ER stress and cytotoxicity on the cells of the trabecular meshwork. Such ER stress may lead to the development of IOP elevation [77-79]. Tg-MYOC^{Y437H} mice were characterized and showed glaucomatous phenotypes, such as IOP elevation, axon degeneration, and the death of RGCs [77]. RGC function, measured by pattern electroretinogram (PERG), was decreased as the mice aged- 40% in 4 months of age and 60% in 12 months of age [77]. In addition, Tyr437His mutant mice showed 20% loss of RGCs in the peripheral retina at 18 months of age, compared to that of wild-type littermates. However, no significant RGC loss was found in the central retina [80].

Neurotrophic factors

Neurotrophic factors are secreted proteins and a variety of NFs are expressed throughout the nervous system. Neurotrophic factors are significant in therapeutic strategies, since they play crucial roles in the survival and neuroprotection of neuronal cells [81-83]. Three major families of NFs are especially relevant to glaucoma: (1) NGF-family, (2) GP130 cytokine, and (3) TGF-beta superfamily [84].

First, the NGF-family includes brain derived-neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin (NT)-3, -4, and -5. Ligands of the NGF-family bind to two different receptors: (1) the tropomyosin receptor kinase (Trk) with high affinity and (2) the p75NT receptor with low affinity. BDNF, NT-4 and -5 bind to TrkB, NGF binds to

TrkA, and NT-3 binds to TrkC. All ligands bind to the p75NT receptor. When a ligand binds to the receptors, downstream signals are activated, such as ERK and mTOR pathways, which promote cell survival and growth. However, signals, activated by p75, result in apoptosis via the activity of Jun N-terminal kinase (JNK) [85].

Second, the GP130 cytokine family includes ciliary neurotrophic factor (CNTF), interleukin-6, and the leukemia inhibitory factor (LIF). The ligand binds to the receptor complex, including GP130, LIF receptor β, and CNTF receptor α. The activation of the receptors promotes cell survival via JAK/STAT signaling [86, 87].

Third, the glial cell-derived neurotrophic factor (GDNF), which belongs to the TGF-beta superfamily, also plays a role in promoting cell survival. TGF-beta superfamily members activate signaling pathways by binding to specific surface receptors. There are two primary types of receptors, type I and type II, which are serine/threonine kinases. Additionally, receptor type III lacks the kinase activity and regulates the availability of ligand to type I and II [203].

GDNF binds to the GDNF family receptor alpha (GFRα) to form receptor complexes. This receptor complexes signal through the transmembrane RET receptor—tyrosine kinase. GDNF binding to a receptor complex activates signaling pathways, such as PI3K and MAP kinase [88].

Brain derived-neurotrophic factor (BDNF) and Retina

BDNF belongs to the NGF family and plays important roles as a mediator of

morphological and synaptic plasticity in the brain [89]. It is known BDNF expression is localized in the retina, and the RGCs and a subpopulation of cells in the inner nuclear layer express BDNF in the mature mouse retina [90]. The expression of TrkB, a BDNF receptor, is expressed in the majority of RGCs, amacrines, and at the vitreal border of the inner nuclear layer in the vertebrate retina. Also, the expression of p75NTR is observed on the Müller cells. The use of neurotrophic factors, such as BDNF, has gained considerable interest for developing strategies for rescuing injured/or degenerating retinas. It is known that retrograde transport of BDNF is interrupted in eyes of experimental glaucoma [91, 92]. Previous studies showed exogenous BDNF prolonged the survival of cultured RGCs [93-95], stimulated regrowth of RGC axons [96], and increased the survival of RGCs [5, 97] *in vivo*. Especially, BDNF delivered via MSCs to glaucomatous eyes with chronic ocular hypertension provided both functional and structural neuroprotection on the host retina and optic nerve [5].

While IOP elevation may interrupt the retrograde transport of BDNF in glaucomatous retinas, endogenous sources within the retina may provide a balance of the expression in the eye. In the retinas of DBA/2J mice, an experimental glaucoma model, the expression of BDNF increases as they age. The elevated IOP induced the expression of BDNF on the Müller glia, while the nerve fiber layer and astrocytes are the locations where heavily concentrated BDNF is observed at all ages [Calkins DS, ARVO 2010]. However, complex pathways involved in BDNF and its receptors—TrkB receptor, truncated TrkB receptor, and p75NT receptor—are not fully understood, and further investigation is required to understand their molecular physiology.

Glial cell-derived neurotrophic factor and retina

Glial cell-derived neurotrophic factor (GDNF) belongs to the transforming growth factor (TGF)-beta superfamily and is a glycosylated homodimer [98]. GDNF binds to the GDNF family receptor alpha (GFRα), a cell surface receptor, to form receptor complexes that signals through the transmembrane RET receptor—tyrosine kinase [88]. Both GFRα and RET receptors were identified on the RGCs, amacrine, and horizontal cells in embryonic chick retina [99]. GDNF binding activates signaling pathways, which include mitogen-activated protein (MAP) kinase, phosphoinositide (PI)-3-kinase, phospholipase C ɣ (PLC-ɣ), and Src-family kinases [88].

GDNF was first isolated from a rat glial cell line and showed the ability to increase the survival of dopaminergic neurons [98]. GDNF is expressed in response to neuronal injury and during retinal development [100-102].

It has been shown that GDNF is secreted by astrocytes [103] and GDNF-receptors are expressed on the RGCs, amacrines, and horizontal cells [99]. As GDNF receptors, Ret is expressed in horizontal, amacrine, or RGCs and not in photoreceptors or Müller cells. GFRα1 expression is found in horizontal, amacrine, and RGCs, GFRα2 is localized in amacrine and RGCs, in the mouse retina. GDNF, like BDNF, is another potent survival factor, since it has been reported to enhance neuronal survival *in vitro* [104], as well as *in vivo* showing promise for the therapy of neurodegenerative disorders or damage [81, 105-108].

Neurotrophic factors and treatment of retinal damage

Elevated IOP is one of the most significant risk factors contributing to glaucoma, and RGC death is a major problem causing irreversible vision loss. It has been reported that ocular hypertension contributes to the deprivation of NFs by blocking retrograde transport [91, 92]. Thus, several kinds of experimental approaches to deliver NFs have been performed thus far. Fibroblast growth factor (FGF) promoted the survival of retinal ganglion cells after optic nerve injury [109, 110]. In addition, administration of purified BDNF [111], ciliary neurotrophic factor (CNTF) [112], and nerve growth factor (NGF) [113] into the eyes of animals with ocular hypertension reduced RGC loss. However, this approach has a limitation that requires repetitive administration of the NFs for long-term protection. Another approach to NF delivery is biodegradable NF-microspheres and gene therapy application. Studies using microspheres manufactured to release GDNF have shown protective effects on the RGCs of degenerating retinas [108, 114, 115]. This method also has shortcomings—burst release or inconsistent speed of release of NF and the release lasts for a relatively short period of time. Gene therapy, another method, using adenoviruses or adeno-associated viruses (AAVs), which encode BDNF [116, 117], GDNF [118], or CNTF [119, 120], has shown a potency to increase RGC survival in animal models of optic nerve transection/or ocular hypertension. This approach has drawbacks, such as immunogenicity of the vectors and toxicity in the host. As an alternative to these limitations discussed above, using stem cells as a cellular vehicle for NF delivery has been suggested and investigated. Previous studies showed bone marrow-derived MSCs, injected into the vitreous cavity, exerted neuroprotective effects on the RGC axons in a rat glaucoma model [4]. In addition, MSCs, engineered to express BDNF, rescued degenerating retinas structurally, as well as functionally [5]. In addition to MSCs, it has been reported that Schwann cells enhanced the survival of RGCs or stimulated axonal regeneration

[121-124], as they produce a variety of neurotrophic factors, cell adhesion molecules, and extracellular matrix, and also they stabilize the elongating axons by utilizing focal tight junctions [125].

The combination of different neurotrophic factors also have been applied and rescued injured or degenerating retinal neurons. Combined injection of BDNF and neurturin as well as BDNF and GDNF showed additive effects on the RGC survival after optic nerve transection. Also, administration of GDNF along with neurturin showed an increased effect on the survival of RGCs [126]. Treatment of basic fibroblast growth factor (bFGF) and epidermal growth factors (EGF) alone enhanced photoreceptor survival *in vitro*, though there was no synergistic effect when combined [127]. The combination of BDNF with CNTF also promoted the survival of rd (retinal degeneration) photoreceptors more effectively than the treatment of either BDNF or CNTF in explants [128]. We suggest that stable and sustained delivery of neurotrophic factors, such as GDNF and BDNF, using a cellular vehicle would be an ideal strategy for long-term neuroprotection to degenerating retina.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs), also called multipotent stromal cells, have the ability to self-renew and differentiate into various mesodermal lineages, such as bone, cartilage, and fat cells [129]. Bone marrow-MSCs can be easily isolated, due to the characteristic of plastic-adherence. For several decades, bone marrow-derived MSCs have been used in numerous studies, as a potential candidate to develop therapeutic strategies on various diseases [130-136]. More importantly, bone marrow-MSCs are a potential candidate for autologous transplantation, which avoids immune response in the host. In addition to

bone marrow, mesenchymal stem cells have been isolated from a variety of regions, such as fetal pancreas [137], liver [138], umbilical cord blood [139], scalp tissue [140], fetal thymus [141], bone and adipose tissue [142], vermiform appendix [143], placenta [144], and endometrium [145].

Bone marrow-derived MSCs are a potential cellular source for stem cell-based therapy, since they have several advantages, including: (1) easy isolation, (2) significant plasticity, (3) no ethical concerns, (4) a potential autologous transplant, and (5) availability of engineering to produce a cellular vehicle for delivering NFs for a long-term period.

Thus, using bone marrow for MSC isolation is a more feasible approach in clinical fields. Many human patients have been treated with bone marrow therapy so far [146, 147]. However, more studies and investigations on MSC research are still needed for further clinical applications.

Bone marrow-MSCs isolated from different rat strains—Fisher, Lewis, Sprague-Dawley and Wistar—have been characterized [148]. However, MSCs from the bone marrow of Brown Norway rats (*Rattus norvegicus*) have not been studied systematically with defining categories. The Brown Norway rat is an inbred rat strain and one of the animal models well-defined genetically, as well as physiologically. Also, the strain has been used in a variety of research fields. Nonetheless, to our knowledge there are no established resources and studies characterizing bone marrow-derived MSCs from Brown Norway rats. Thus, isolation and systematic examinations of MSCs from this strain are required to broaden the availability of cell lines for autologous or syngeneic transplant to develop experimental strategies for neurodegeneration.

Cellular-based retinal therapies

The eye is an immune privileged site [149, 150] and provides tolerant environments for cell transplant, minimizing the risk of immune rejection. Thus, the eye is a more suitable region for the application of cell-based therapy compared to other body parts, where the immune system is active in response to foreign materials. Cell-based therapy has been developed widely and recognized as very promising for the cure of degenerative retinal diseases. Currently, the available approaches for cell-based therapy can be categorized into two major strategies—cell replacement and neuroprotection—investigated by cell transplantation.

A variety of cell sources have been developed and investigated to rescue degenerating retinas *in vivo*. Human embryonic stem cell-derived retinal pigment epithelium (RPE), transplanted into the subretinal region of a Royal College of Surgeons rat, improved visual performance [151]. In addition, differentiated human fetal RPE [152], brain stem cells [153], bone marrow–derived stem cells [2, 4, 154, 155], retinal stem cells [156, 157], Schwann cells and fibroblast [78], retinal sheet [158, 159], and induced pluripotent stem cells (iPSCs) [160-162] have been used *in vivo* studies. All showed rescue effects to some extent. However, none of these studies provided sufficient evidence for long-term survival of transplant, complete retinal integration, and morphological preservation, as well as the functional restoration of the host retina for a long-time period.

Stem cells have been extensively investigated and proposed as a powerful source of cell transplantation, due to the capacity of self-renewal and multi-potency. Mesenchymal stem cells (MSCs), among a variety of types of stem cells, possess great advantages for

success in cell-based therapy. They are robust, proliferate well by providing large quantities of cells, differentiate into multiple lineages of cell types [129], survive well in the host retina, release trophic factors, contain immunomodulatory functions, and are a potential candidate for autologous transplant [129, 163-166].

Previous studies showed bone marrow-derived MSCs injected into the vitreous cavity exerted neuroprotective effects on the RGC axons in a rat glaucoma model [4]. MSCs have shown morphological neuroprotective effects on the survival of RGCs/or their axons following intravitreal transplantation into the glaucomatous eyes [3, 4]. Supplying neurotrophic factors to the retina in acute or chronic models of glaucoma have shown a potential approach to protect damaged retinas [108, 114, 119]. As a vehicle to deliver neurotrophic factors into the retina, mesenchymal stem cells (MSCs) could play an effective role in the eyes with experimental glaucoma. In addition, MSCs, engineered to express BDNF, rescued degenerating retinas structurally, as well as functionally [5].

Viral constructs

The use of viral vectors is a very powerful approach to deliver genes of interest into cells or tissues. Viral vectors, including lentivirus, recombinant adenovirus, and adeno-associated virus (AAV), possess high efficiency to deliver the gene. Therefore, they are commonly used for gene therapy [167-171].

The lentiviral vector (LV) is a potent and efficient construct to deliver genes into nondividing and dividing cells. Also, lentiviral constructs are a self-inactivating vector, possess the capability to integrate into the genome of host cells, and result in sustained expression of the transgene [172-174]. Lentiviral vectors have been studied for use in gene

therapy for the retina [175-182]. It was shown that gene transfer was successful as a transducer for both retinal pigment epithelium and photoreceptors in the eyes [170].

Gene transfer via lentiviral vectors protected retinal degeneration in the rat model of Retinitis pigmentosa [179] and preserved cone function in the mouse model of Leber congenital amaurosis [181]. Also, the survival of injured RGCs was enhanced in rats with axotomy after injection of LVs expressing ciliary neurotrophic factor (CNTF) [180]. However, concerns exist with applications of these vectors, due to low efficiency of viral infection to mature retinal neurons *in vivo* [183] and transient toxicity on the retinas [184], when used directly into the eyes.

Lentiviral vectors that delivers BDNF or GDNF genes have been developed also and used for the studies of neural repair. BDNF gene transfer via lentiviruses provided neuroprotection in a model of excitotoxicity [204] and showed potential rescue effects on an injured spinal cord [205]. Delivery of the GDNF gene is also neuroprotective on a model of Parkinson's disease [208], lesioned motorneurons [207], and excitotoxicity [206].

To overcome the limitations stated above, lentiviral vectors, instead, can be used to infect stem cells *ex vivo* for cell-based therapy [185]. These constructs are a very powerful and desirable vehicle to deliver genes into the cells, since they have excellent efficiency for cell infection and can be manufactured on a large scale for use in therapeutic treatments [186].

Retinal functional measurement

Pattern electroretignogram (PERG) is a non-invasive method that allows assessment of visual eye function. Patterned light stimuli, which consist of black and white checkerboards or gratings, are provided at a constant luminance. The retinal activity, in

response to the stimuli, is noted by a recording electrode lying on the surface of the cornea [187]. The source of response signals originates from the retina's RGCs. Patterned gratings produce lateral inhibition mediated by horizontal cells. This causes differential activation of the dendritic field on RGCs, resulting in the local activity of RGCs summed at the recording electrode. Since the retinal potential measured by PERG is driven by cones, focal, and post-receptoral, the amplitude detected is very small, compared to the conventional ERG [187]. The retinal potential of PERG is displayed as a waveform, which consists of an initial negative component (N), followed by a positive peak (P), and another large negative component (N). For humans, each component is indicated as N35, P50, and N95, respectively, reflecting their latency. The amplitude between peaks and troughs is measured for functional evaluation, in general [188].

The usefulness of PERG, as a tool to monitor the dysfunction of RGCs in DBA/2J mice, was demonstrated [189]. DBA/2J mice showed a progressive decrease of PERG amplitude after 3 months of age and the amplitude reached the noise level by 11 months [56]. It has been reported that conventional full-field ERG is not significantly affected by inner retinal dysfunction on the DBA/2J mice [189].

Different from PERG, conventional ERG utilizes a bright flash of light to obtain a response from all retinal neurons. Typical ERG traces include different wave components. The a-wave indicates the function of photoreceptors in the outer nuclear layer [190]. The b-wave indicates the function of inner retinal neurons. Oscillatory potentials, included in the b-wave, indicates the activity between the neurons of INL and RGCs [191]. The usefulness of ERG has been demonstrated on evaluating retinal ischemia [191].

In addition to these ERG measurements, pupillary light reflex (PLR) can be used to

evaluate retinal and optic nerve function. Light stimuli elicit PLR that induces iris constriction and change of pupil diameter in response to the light. The reflex pathway begins with retina, the signal then travels from the retina to optic nerve, Edinger-Westphal and pretectal olivary nuclei, and ciliary ganglion via oculomotor nerve, in sequence. Lastly, ciliary nerve leaving the ciliary ganglion constricts the iris muscle. Pupillary light reflex is mediated by intrinsic photosensitive retinal ganglion cells (ipRGCs) which contain photopigment, melanopsin. As, such this non-image-forming visual response is independent of rod and cone photoreceptors [192, 193]. Pupillometry has been utilized to characterize healthy eyes of rat and mouse [194, 195] and showed significant defects in hypertensive eyes [196, 197] and in ischemic retina [198]. In addition, pupillometry has been used to evaluate functional rescue after ocular transplant to a rat model of photoreceptor degeneration [199], and used as a clinical examination of glaucoma [200-202].

Summary

Mesenchymal stem cells are a potential candidate for autologous transplants and is of great significance in clinical therapies. Investigating stem cell-based therapy to develop neuroprotective strategies is essential to rescue a neurodegenerative eye, since there are no remedies to cure glaucoma and stem cells have been shown to be powerful to save degenerating retinas morphologically and functionally. The studies discussed in this dissertation provide evidence of a new MSC line, isolated from the Brown Norway rat, for identification of bona fide MSCs and potency as therapeutic resources. In addition, we assess

the potential effects of engineered MSCs, as allografts, on two different transgenic mouse models of glaucoma using intraocular transplantation of the MSCs.

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CHAPTER 2: BONE MARROW-DERIVED MESENCHYMAL STEM CELLS STIMULATE NEURITE OUTGROWTH FROM ADULT HIPPOCAMPAL PROGENITOR CELLS

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Abstract

Bone marrow-derived mesenchymal stem cells (MSCs) have recently emerged as beneficial cellular vehicles for nervous system rescue and repair. A better understanding how MSCs are involved in mediating neural repair will facilitate development of novel therapeutic strategies. Mesenchymal stem cells are ideal candidates for cell-based therapies because of their ease of isolation, capacity of self-renewal and phenotypic plasticity. There is an increasing need for additional studies of MSCs since they are a potent cell source for cell transplants to overcome many diseases. Variations exist on MSC characteristics between different strains, and little has been studied in regards to bone marrow-MSCs from Brown Norway rats. In the present study we aimed to isolate and characterize bone marrow-MSCs from this strain, and more importantly we studied the capacity of MSCs to influence the differentiation of adult hippocampal progenitor cells (AHPCs). These MSCs have been subcultured up to 20 passages and they showed stable and consistent growth rate. MSCs were identified using a panel of phenotypic markers that are positive/or negative to rat MSC.

MSCs retained short population doubling time (PDT) and showed very high proportions of BrdU-incorporation, an indicator of proliferative cells. In addition, MSCs showed good cell viability, evaluated by propidium iodide (PI) staining, and possess the capacity of multipotent differentiation (adipogenesis and osteogenesis). We also found endogenous expression of a neural marker, nestin, in a small proportion of the MSCs. In order to explore the potency of MSCs to promote regeneration and repair of nervous systems, we examined whether contact co-culture (CCC) of MSCs influences AHPC differentiation. Our results revealed that MSCs did not significantly affect the proportions of proliferation and neuronal-/and glial-lineage of AHPC differentiation. However, both CCC and non-contact co-culture (NCCC) significantly promoted neurite outgrowth on neuronal AHPCs and also notably increased branching processes on oligodendrocytic AHPCs.

Our study indicates that the MSCs are a new useful resource for stem cell research, including therapeutic applications as a syngeneic transplant to Brown Norway rats. More interestingly, the ability of MSCs to promote morphological differentiation on neuronal and oligodendrocytic AHPCs would be another attractive value to develop strategies for cell-based therapies to rescue degenerative nervous systems.

Keywords Brown Norway rat, bone marrow-mesenchymal stem cells, growth, viability, multipotent differentiation, co-culture with AHPCs, neurite outgrowth

Introduction

Mesenchymal stem cells (MSCs) have become an important cell source for treatment of neurodegenerative conditions as well as nerve repair strategies. Gaining a better understanding of how MSCs mediate neural repair will benefit the development of novel therapeutic strategies [1-7]. Multipotent MSCs have the ability to self-renew and can differentiate into various mesodermal lineages such as bone, cartilage, and fat cells [8]. Bone marrow-derived MSCs can be readily isolated due to their characteristic adherence to tissue culture plastic surfaces. Importantly, bone marrow-MSCs are a potential candidate for autologous transplantation, thus avoiding an immune response in the host. In addition to bone marrow, mesenchymal stem cells have been isolated from a variety of tissues such as fetal pancreas [9], liver [10], umbilical cord blood [11], scalp tissue [12], fetal thymus [13], adipose tissue [14], vermiform appendix [15], placenta [16], and endometrium [17]. However, MSC isolation from bone marrow is a relatively common procedure and is clinically relevant [18, 19].

Bone marrow-MSCs isolated from different rat strains- Fisher, Lewis, Sprague-Dawley and Wistar- have been well characterized [20]. However, MSCs from the bone marrow of Brown Norway rats (*Rattus norvegicus*), have not been studied systematically. Brown Norway rats are a relatively common animal model used for biomedical research. They are well-defined genetically, physiologically, and behaviorally [21-26]. There are, to our knowledge, no established resources and studies performed on bone marrow-MSCs from Brown Norway rats. Thus, the isolation and systematic examination of MSCs from this strain is required to broaden the availability of cell lines for autologous or syngeneic transplants for further development of experimental strategies for neurodegeneration.

In this study we have characterized bone marrow-MSCs from Brown Norway rats. Three criteria were used to define the MSCs; 1) adherence to tissue culture plastic, 2) expression of specific surface antigens, and 3) multipotent differentiation potential [27]. In addition to the characterization of MSCs, we also examined the cell growth, proliferation, and viability of MSCs. To gain a better understanding of the ability of MSCs to promote regeneration and repair of the nervous system, we investigated whether MSCs have the capacity to stimulate neurite outgrowth from adult rat hippocampal progenitor cells (AHPCs). In this study we demonstrate that MSCs isolated from the bone marrow of Brown Norway rats are a pure MSC population and capable of differentiating into adipogenic and osteogenic lineages. The MSCs showed consistent cell growth and proliferation, as well as good viability through long periods of subculture. Moreover, we demonstrated the capability of MSCs to promote neurite outgrowth from TuJ1-IR AHPCs and to enhance oligodendrocytic differentiation of AHPCs. These results provide additional support for the use of MSCs is a potent resource for the development of strategies for cell-based therapies to rescue degenerating nervous systems.

Materials and Methods

Animals

Male and female Brown Norway rats, obtained from Charles River Labs, at 6 weeks of age were used for the isolation of bone marrow. The animals were kept in a constant environment (temperature: 22°C; humidity: 20 %; 14/10-hour light-dark cycle) with food and water provided ad libitum until bone marrow was isolated. The rats were allowed to rest for

at least 7 days after arrival from the supplier to let them recover from transportation and adapt to the new environment before harvesting of bone marrow. All animal procedures for this study adhered to the provisions of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and had the approval of the Iowa State University Institutional Animal Care and Use Committee, and were performed in accordance with committee guidelines.

MSC isolation and culturing

The rats were euthanized with isoflurane and then the femora and tibiae were dissected. These bones were placed in a 50 ml conical tube filled with ice-cold culture media [(CM; alpha minimum essential medium (αMEM; Gibco BRL, Gaithersburg, MD) supplemented with 20 % fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 4 mM L-glutamine (Gibco BRL, Gaithersburg, MD), and antibiotic-antimycotic (Invitrogen, Carlsbad, CA)]. For each bone, epiphyses were removed and the marrow was forced out into a fresh 150 mm culture dish using a syringe and 23-gauge needle filled with 3 ml CM. The marrow suspension was expelled onto a 70 μm filter pre-wetted with CM and transferred into a T75 flask with 20 ml CM. The cell suspension was maintained in a culture incubator (37°C, 5% CO_2 / 95% humidified air atmosphere). 48 hours after harvest, spindle-shaped cells adhered to the flask and media was changed with fresh CM after washing with phosphate-buffered saline (PBS; Gibco BRL, Gaithersburg, MD). MSCs isolated from male and female rats were cultured separately as different cell lines. MSCs were fed with CM twice a week. When the MSCs were about 80 % confluent, subculturing was performed.

Population doubling time

To evaluate the growth of MSCs, we calculated population doubling time (PDT) using the formula PDT = 2 x t x Ni/No (t: the time required to reach 80% confluency, hr; Ni: the initial number of cells; No: the final number of cells) [28]. MSCs at passages 1, 4, 9, and 15 were investigated (N=3).

5-bromo-2-deoxyuridine (BrdU) assay

The proliferation of MSCs was evaluated by incorporation of BrdU (5-bromo-2-deoxyuridine) into the cells at several passages that correspond to that of the PDT analysis, (i.e. passages 1, 4, 9, and 15). MSCs were plated onto 12 mm cleaned glass coverslips at approximately 30% confluency with maintenance media (MM), and the following day BrdU solution was added (5 μM BrdU in MM). After 24 hours, MSCs were fixed in 4% paraformaldehyde in 0.1 M PO_4 buffer, pH 7.4. Fixed cells were then rinsed in PBS (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) and incubated in 2N HCl for 15 min followed by 0.1 M sodium borate solution (pH 8.5) for 5 min. After washing with PBS, MSCs were incubated in blocking solution [5% normal donkey serum, 1% bovine serum albumin (BSA; Sigma), and 0.1% Triton X-100 (Fisher Scientific) in PBS] for 90 minutes. A primary antibody (see Table 1) against BrdU was used. To identify cells that incorporated BrdU, MSCs were incubated in primary antibody overnight at 4°C in a humid chamber, washed in PBS, and incubated in Cy3-conjugated secondary antibody for 1.5 hours in the dark. The cells were then rinsed and nuclei stained with 4', 6-diamidino-2-

phenylindole, dilactate (DAPI, 1:2,000). Preparations were mounted on glass slides with Vectashield mounting media (Vector laboratories, Burlingame, CA).

Propidium iodide (PI) staining

To investigate the viability of MSCs, PI (Invitrogen, Carlsbad, CA; final concentration 1.5 µM) was added to the culture dishes in the dark for 20 min, at 37^{o}C. Cells were then fixed in 4% paraformaldehyde in 0.1 M PO_4 buffer, pH 7.4 and stained with DAPI. As a positive control, a group of cells were incubated with 70% ethanol for 2 minutes and incubated with PI in the same conditions. Under this control condition all MSCs were PI-labeled.

Adipogenesis

A mesenchymal stem cell Adipogenesis kit (Cat. No. SCR 020; Millipore, Billerica, MA) was used to generate adipocytes from the isolated rat MSCs. The induction protocol as specified in the datasheet was applied. Briefly, MSCs were plated at a density of 60,000 cells per well in a 24-well culture plate. When the cells were 100 % confluent, adipogenesis induction medium was added into the wells. Induction/and maintenance medium was changed every two days for 21 days. MSC cultures were fixed in 4% paraformaldehyde in 0.1 M PO_4 (pH 7.4) for 30 minutes at room temperature and rinsed. Oil Red O Solution was added for 50 min to stain adipocytes containing lipid droplets. Cell nuclei were stained with

hematoxylin solution incubating for 15 minutes. Astrocytes subjected to the same conditions were used as a negative control.

Osteogenesis

MSCs were induced to differentiate into osteogenic lineages. A mesenchymal stem cell Osteogenesis kit (Cat. No. SCR 028; Millipore) was used for induction as per the protocol specified in the datasheet provided. Briefly, each well of a 24-well plate was coated with vitronectin and collagen in 1X PBS to yield a final concentration of 12 μg/mL for each ECM molecule and MSCs plated at a density of 60,000 cells per well. When the cells were 100 % confluent, osteogenesis induction medium was added into the wells. Induction medium was changed every 2~3 days for 14 days. Osteocytes were fixed in iced cold 70% ethanol for 1 hour at room temperature. Alizarin Red Solution was added for 30 minutes in order to stain osteocytes containing calcium deposits. Astrocytes subjected to the same conditions were used as a negative control.

Co-Culture of MSCs with AHPCs

Adult hippocampal progenitor cells (AHPCs, provided by F. Gage, Salk Institute, La Jolla, CA), originally isolated from the brains of adult Fischer 344 rats as reported by Palmer and colleagues [29], were used to perform co-culture with MSCs. The culture groups consisted of 1) AHPC only, 2) MSC only, 3) Contact co-culture (CCC), and 4) Non-contact co-culture (NCCC) of AHPCs with MSCs. First, MSCs were plated on the glass coverslips

coated with poly-L-ornithine (100 µg/ml in sterile water) and laminin (10 µg/ml in Earle's Balanced Salt Solution (EBSS) (PS-LAM substrates) at an initial density of 7,000 cells per well of 6 well culture plate. For the NCCC, we used 0.4 µm semi-porous membrane inserts (Corning,Inc., Corning, NY) to separate the MSCs from AHPCs cultured on the surface of 6 well culture plate. The membrane inserts were placed inside the well and then filled with co-culture media. Then, MSCs were plated onto the insert well of the plate. The following day, AHPCs were plated onto the same culture plate at 20,000 cells per well. The co-cultures were maintained in a mixed medium, co-culture media, consisting of AHPC differentiation media in a 7:3 mixture with MSC growth media (with 10% FBS) (referred to as co-culture media). As controls, AHPCs and MSCs were plated in the same co-culture medium at the same density. Cells were maintained at 37^{o}C in a 5% CO_2/95% air atmosphere in culture medium. After 7 days culture, the cells were fixed and immunostained as described in the following immunocytochemistry section.

Immunocytochemistry

To characterize MSCs, we performed immunocytochemistry with a panel of cell-type specific antibodies. A Rat Mesenchymal Stem Cell Characterization kit (Cat. No. SCR018; Millipore) was also used for analysis. For immunolabeling, MSCs were fixed in 4% paraformaldehyde in 0.1 M PO_4 buffer, pH 7.4. Fixed cells were incubated in blocking solution containing 5% normal donkey serum, 0.4% bovine serum albumin (BSA; Sigma), and 0.2% Triton X-100 (Fisher Scientific), followed by incubation with primary antibodies overnight at 4°C. Primary antibodies and their dilutions are listed in Table 1. After rinsing in

PBS, cells were incubated in the secondary antibodies conjugated to Cy3 diluted at 1:500 (Jackson ImmunoResearch, West Grove, PA). Cell nuclei were stained with 4', 6-diamidino-2-phenylindole, dilactate (DAPI), diluted at 1:2,000 in PBS and applied for 30 minutes. Preparations were rinsed and then mounted onto microscope slides using an antifade mounting medium (Gel Mount; Biomeda Corp., Foster City, CA). Negative controls were performed in parallel by omission of the primary antibodies. No antibody labeling was observed in the controls.

Quantification of neurite outgrowth

To perform a quantitative analysis on the neurite outgrowth of AHPCs, we assessed the number of neurite branches on TuJ1-immunoreactive (TuJ1-IR) AHPCs. The extent of neurite arborization was measured using a Sholl analysis [30, 31] plugin to NIH ImageJ. The concentric circles plugin was used to aid in the Sholl analysis by drawing concentric circles around the cell. Three concentric circles with radii 10, 20, and 30 μm from the center of the cell soma were drawn. The number of intersections between the circles and neurites were then manually counted. Analysis was performed in masked fashion.

Imaging and statistics

Phase contrast images were taken using a Nikon Diaphot inverted microscope with a CCD camera (Megaplus; Model 1.4; Kodak Corp., San Diego, CA) connected to a frame grabber (Megagrabber; Perceptics, Knoxville, TN, in a Macintosh computer; Apple Computer, Cupertino, CA) using NIH Image 1.58VDM software (Wayne Rasband, National

Institutes of Health, Bethesda, MD). Images of MSCs labeled with the antibodies were captured using an upright fluorescence microscope (Nikon Microphot FXA) equipped with a Retiga 2000R digital camera controlled by QCapture software (QImaging, Surrey, British Columbia, Canada). Figure plates were prepared using Photoshop CS2. Data were reported as means ± S.E.M. Statistical analysis was performed using GraphPad PRISM (ver. 3.0). All tests were two-tailed tests and P values less than an alpha of 0.05 were considered significantly different.

Results

Isolation and characterization of Brown Norway rat MSCs

Culture of MSCs: Mesenchymal stem cells (MSCs) from male and female Brown Norway rats were isolated from the bone marrow by the characteristic of adhering to a plastic culture surface. The adherent MSCs were cultured as a monolayer and passaged when they reached 70~80% confluency. As illustrated in Figure 1, the MSCs showed a typical spindle-shape and fibroblast-like morphology. At early passage, small and slender MSCs were predominantly observed in the population. At later passages, we observed a relatively larger ratio of cells with large and flattened morphology compared to that of early passage cells (Fig. 1).

Characterization of MSCs: Characterization of the bone marrow-derived cells was performed by immunocytochemistry with rat MSC-specific antibody markers. A panel of negative and positive antibodies for rat MSCs was used (Table 1). After culturing for 4 or 5

passages, the vast majority of the adherent bone marrow-derived cells were specifically labeled with antibody markers for MSCs (CD29, CD51, CD54, CD90, fibronectin, and collagen type I) (Fig. 2A - F). Furthermore, no MSCs showed specific immunoreactivity for the negative markers (CD11b, CD14, CD44, and CD45) (Fig. 2G-J). (See also Supplementary Fig. 1. for the characterization of female MSCs.)

In addition to the screening with MSC-specific markers, we also examined the populations with neural markers to examine potential expression of endogenous neural-lineage markers. About 30% of MSCs were nestin-IR (Suppl. Fig. 2.) and no specific staining was found for TuJ1, MAP2ab, or GFAP antibodies (Images not shown).

MSC growth rate and proliferation

To investigate the growth of MSCs quantitatively, we assessed population doubling time (PDT) for the MSCs isolated from both the male and female rats. The growth and proliferation of MSCs were analyzed at passages 1, 4, 9, and 15 (Table 2). MSCs from female rats (♀) showed values of PDT (hr), 23.74 (± 7.712), 37.01 (± 4.925), 25.76 (± 7.072), and 28.73 (± 7.743) at P1, 4, 9, and 15, respectively. MSCs from male rats (♂) also had a similar range of growth rates with that of MSCs (♀); 17.68 (± 4.137), 29.67 (± 2.164), 25.5 (± 2.629), and 23.43 (± 2.563) at P1, 4, 9, and 15, respectively. There were no significant differences between the different passages/or between MSCs, isolated from male versus female Brown Norway rats (P value > 0.05). In addition, a BrdU assay was performed to examine the proliferation of MSCs with increasing passage number (Table 3). MSCs were exposed to 5 μM BrdU for 24 hours. More than 80% of MSCs were BrdU-immunoreactive

(IR) at most passages. A significantly lower percentage (54.76%) of BrdU-labeled cells was observed for the MSCs (♂) at P4, although the BrdU percentages were essentially equal between the MSC populations at all other time points.

Cell Viability

To investigate the viability of MSCs while maintained in culture, we performed a cell viability staining assay using propidium iodide (PI), a DNA-binding probe. Because of the permeability of PI to cells with compromised membrane integrity, we can differentiate unhealthy or dead cells from healthy and viable cells by the fluorescence of PI which binds to DNA in the nucleus. PI was added directly to the culture dishes with final a concentration 1.5 µM for 20 min. MSCs maintained under normal growth conditions were not labeled by the PI (0% PI-labeled, Supp. Fig. 4A, N=3). However, PI-bound MSCs were shown as a positive control (100% PI-labeled following ethanol treatment; Suppl. Fig 3B; N=3).

Differentiation of MSCs into mesodermal lineages

The multipotential nature of the MSCs was investigated by examining their ability to differentiate into adipogenic and osteogenic lineages. MSCs differentiated into adipocytes 21 days after adipogenic induction. Lipid droplets in adipocytes derived from MSCs following induction were stained with Oil Red O solution (Fig. 3. A and A').

MSCs subjected to osteogenic induction conditions for 14 days were visualized with Alizarin red solution. Amorphous deposits of calcium were stained red, demonstrating

osteogenic differentiation ability of the MSCs (Fig. 3. B and B'). For both differentiation paradigms (adipogenic and osteogenic) astrocytes were used as a negative control and were subjected to the induction protocols and resulted in no Oil Red O or Alizarin red staining, respectively (Suppl. Fig. 4. A'' and B''). Taken together, these results indicate that we have successfully isolated populations of multipotent Brown Norway rat bone marrow-derived MSCs.

Co-culture of MSCs with AHPCs

To examine the possibility that MSCs can influence the proliferation and differentiation of adult neural progenitor cells, we established co-cultures of adult hippocampal progenitor cells (AHPCs) with MSCs. To delineate possible contact-mediated and/or soluble inducing activities associated with the MSCs, the AHPCs were differentiated in parallel under three different culture conditions: (1) AHPCs cultured alone, (2) AHPCs cultured with MSCs in noncontact co-culture conditions (NCCC), and (3) AHPCs co-cultured in physical contact with the MSCs (contact co-culture condition, CCC). The AHPCs express green fluorescent protein (GFP) which facilitated their identification when co-cultured with the non-GFP-expressing MSCs (Fig. 4). After 7 days, cultures were fixed and immunostained to examine cell proliferation (BrdU-IR) and differentiation. A BrdU analysis revealed no significant differences in the overall percentages of AHPCs immunoreactive for the BrdU antibody (P value $>$ 0.05; Table 4.). To investigate differentiation, the percentages of AHPCs immunoreactive for neuronal (TuJ1-IR) or oligodendrocyte (RIP-IR) markers was determined. When cultured alone, ~21% of the AHPCs were TuJ1-IR and ~59% RIP-IR

(Table 4.). When co-cultured with MSCs under noncontact conditions (NCCC) ~23% of the AHPCs were TuJ1-IR and ~29% RIP-IR. When AHPCs were co-cultured in physical contact (CCC) with MSCs ~19% were TuJ1-IR and ~55% RIP-IR. No significant differences in AHPC differentiation into TuJ1-IR neurons was observed across the three culture conditions. Although the percentage of AHPCs immunolabeled for RIP was on average lower in the NCCC condition, there was no significant difference on the percentages between the culture groups (P value $>$ 0.05; Table 4.).

Morphologically we noted discernable differences on TuJ1- and RIP-IR AHPCs in both of the co-culture groups (CCC and NCCC) when compared to the AHPCs cultured alone. Co-culture with MSCs stimulated neurite outgrowth of neuronal AHPCs (TuJ1-IR). TuJ1-IR AHPCs, in both NCCC and CCC conditions, showed longer and highly branched neurites when compared to the AHPC only condition (Fig. 5.). Quantitative assessment was performed by Sholl analysis as illustrated in Figure 5D. There were significant differences in the number of intersections at a radius of 20 μm (AHPC only vs. NCCC and AHPC only vs. CCC; P value $<$ 0.05). These results indicate that MSCs and or MSC-derived factors played a significant role in the morphological differentiation of AHPCs by promoting neurite outgrowth during the co-culture conditions. Furthermore, physical contact (CCC) between the MSCs and AHPCs during co-culture resulted in thicker neuronal processes and increased complexity, compared to that of the NCCC. In addition to influencing neurite outgrowth from TuJ1-IR cells, MSC co-cultures also resulted in more highly branched RIP-IR cells. However, we could only analyze the morphology of RIP-IR AHPCs qualitatively, because the processes of RIP-IR cells were usually too close or overlapping to be distinguishable from those of adjacent cells. Both in NCCC and CCC conditions, RIP-IR cells showed more

highly branched morphologies and a larger area of processes than that of the AHPC only group. In addition, the thickness of primary processes in CCC group appeared to be thicker after co-culture, compared to those of the other groups (Indicated with an arrow head; Fig 6.).

Discussion

Bone marrow-derived MSCs possess considerable potential towards development of cell-based therapeutic strategies. The present study isolated and characterized bone marrow-derived MSCs isolated from male and female Brown Norway rats (♂ and ♀), a commonly used strain for biomedical research [32-37]. MSCs were successfully isolated from the bone marrow and were cultured for 20 passages displaying stable and consistent growth rates. Immunostaining with a panel of MSC positive- and negative- antibody markers demonstrated that the identity of these populations of cells are consistent with MSCs, lacking hematopoietic cell lineages. The PDTs for these MSCs was about a day, indicating a relatively rapid cell proliferation rate. Furthermore, BrdU assay indicated that most MSCs maintained a proliferative capacity throughout the passages examined. When maintained under optimal growth conditions, cell viability was high, with little PI staining indicative of cell death. In addition, the multipotential nature of these MSCs was demonstrated based on their ability for adipogenesis and osteogenesis. The isolation and characterization of these Brown Norway rat MSCs will broaden the availability of MSC lines for autologous and syngeneic transplants towards development of experimental strategies for treating neurodegenerative conditions.

When co-cultured with adult hippocampal progenitor cells (AHPCs), the MSCs provided significant stimulation of neurite outgrowth. The MSC-associated activity is in part likely mediated via soluble cues.

The Brown Norway rat MSCs isolated and characterized in this study initially were a somewhat heterogenous population showing typical morphologies, consisting of spindle-shaped and fibroblast-like cells as reported previously [42]. The fibroblastic cell morphology became more prominent over time upon continued subculturing. The morphological characteristics of these MSCs are consistent with other rodent strains of MSCs [20, 28, 41].

Cell phenotyping was conducted using a panel of MSC positive- (fibronectin, collagen type I, CD29, CD54, CD51, and CD90) and negative- (CD11b, CD14, CD44, and CD45) antibodies for rat MSCs. In all experiments the MSCs were immunoreactive for the positive markers and no detectable immunolabeling for the negative MSC markers was observed, suggesting that the population lacked of hematopoietic lineage cells, consistent with a highly pure population of MSCs.

The growth and proliferation of MSCs were studied and compared between cells isolated from male and female donor rats to examine the possibility of intrastrain sex differences. With increasing passages, MSCs showed some variability in population doubling time (18 - 37 PDT (hours)) though the average PDTs were not significantly different from early to late passages. Furthermore, no significant differences of PDTs were found between male- and female-MSCs as well as between early and late passages. The results of BrdU assay also suggest that most MSCs were in actively replicating state. This property would be a benefit to meet the needs of generating a large number of cells for scientific research and

preclinical applications. Unexpectedly, a low percentage of BrdU-IR cells was observed at P4 (♂). Previous studies reported reduced proliferation and/or growth-arrest in rat MSCs at passage 4 or 5 [43-45]. Population doubling time at P4 (♂) in the present study, however, was not significantly different from other passages and only the ratio of BrdU-IR cells decreased. A possible reason may be that a larger proportion of MSCs may linger in G2, M, or G0 phases compared to cells at other passages. It is also possible that the time window of BrdU exposure for the MSCs was somewhat narrow at P4 (i.e. MSCs were exposed to BrdU for 24 hours during 5 days culture (t=5 at P4 (♂); PDT = 2 xt xNi/No) at P4). This could possibly result in limited incorporation of BrdU into the sub-population in S phase during this period. The PDT value of these Brown Norway rat MSCs was relatively short compared to those of other strains of rat (Fisher, Lewis, Sprague-Dawley, and Wistar) which showed approximately 2-4 days PDT [20]. However, consistent with our data, Karaoz and colleagues [28] reported 19-41 hours PDT for Wistar rat MSCs. Nonetheless, it is clear that growth characteristics of MSCs, in fact, can vary depending on the species, strains, passages, and the regions from which cells were isolated [11, 41, 46] . It is possible that such differences may be due to animal age, techniques used for cell isolation and culture, as well as general health conditions between individual animals.

The multipotential ability of these MSCs was demonstrated by their differentiation potential into mesodermal lineages. Following the appropriate induction protocols, the MSCs had the ability to differentiate into adipocytes and osteocytes *in vitro*. A growing body of literature indicates that MSCs possess phenotypic plasticity and are able to generate myoblasts, tendon/ligament fibroblasts, adipocytes, osteocytes, and chondrocytes [8]. Karaoz et al. [28] demonstrated endogenous expression of osteo-, myo-, and neuro-genic markers,

which supports the plasticity of rat MSCs to differentiate into various kinds of cell types. A small proportion of Brown Norway rat MSCs were immunolabeled with a nestin antibody, a neural stem cell marker. A complex filamentous network of immunolabeling was observed in these MSCs, consistent with nestin intermediate filament labeling. Nevertheless, the ability of MSCs to transdifferentiate into neural cells remains a complicated and controversial issue requiring additional studies [75, 76].

A clearly emerging theme for use of MSCs as cellular vehicles for neural repair is their neurogenic and neuroprotective activities. MSCs have the ability to synthesize and secrete a variety of biomolecules such as neurotrophic factors, cytokines, and growth factors [47, 48]. A number of studies have demonstrated that such factors can enhance neural cell proliferation, differentiation, and survival [49-55]. Furthermore, MSCs have been shown to secrete neurogenic factors including brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and neurotrophin-3 (NT-3). [47, 56, 57]. In the present study, we investigated the ability of these MSCs to influence the differentiation of adult rat hippocampus derived progenitor cells (AHPCs). These multipotent AHPCs are capable of differentiating into neurons, oligodendrocytes and astrocytes [58-60]. Two types of co-cultures were established to exam cell-cell associated activities, as well as secreted soluble factors from the MSCs. No significant differences in the proportion of AHPC differentiation towards a neuronal (TuJ1-IR) or glial (RIP-IR) cell type were observed in the co-cultures of MSCs with the AHPCs. However, morphological differentiation of both neuronal and oligodendrocyte-like AHPCs was evident. Both co-culture configurations (NCCC and CCC) resulted in significant increases in neurite outgrowth and complexity on neuronal AHPCs (TuJ1-IR cells) when

compared to AHPCs differentiating on their own. We found the expression of TrkB, a receptor for BDNF, on AHPCs (data not shown). This would support our results, i.e. increased neurite outgrowth from AHPCs when co-cultured with MSCs. We, therefore, presume that bioactive BDNF secreted from MSCs binds to TrkB receptors to stimulate neurite outgrowth of neuronal AHPCs. Neurotrophic factors such as BDNF induce neurite outgrowth from neuronal cells and neural progenitors [61, 62]. MSCs also express a variety of neuro-regulatory molecules, including neurite-inducing factors and axon guidance molecules [77]. Further studies will be required to examine what other types of receptors for biomolecules are expressed on AHPCs. Interestingly, when co-cultured in direct contact with the MSCs (CCC) the AHPCs displayed even more increased complexity of neurites than that of NCCC. Thus, it is likely that cell-cell interactions mediated by cell adhesion molecules and/or extracellular matrix molecules (ECM) also play important roles in the MSC neurite outgrowth promoting activity [63-66]. In the present study, we showed that the expression of ECM molecules (fibronectin and collagen type I) and CD29 (integrin β_1) on MSCs. It is well-known that fibronectin and collagen type I interact with integrin $\alpha_5\beta_1$ and $\alpha_2\beta_1$, respectively [67]. Moreover, our previous study reported the expression of integrins (α_2, α_5, and β_1) and ECM molecules (fibronectin and laminin) on the rat adult brain progenitors [60]. Thus, it is possible that physical contact between MSCs and AHPCs would allow cellular interactions mediated through integrin-ECM signaling to stimulate neurite outgrowth. Interactions between the ECM and integrins activate signaling pathways that modulate the dynamics of the cytoskeletons [68], and the changes of cytoskeletal proteins involved in microtubule and actin filaments contribute to the formation and regulation of neurite outgrowth [69-71].

MSCs possess considerable therapeutic potential due to a number of advantages, including relative ease of isolation, plasticity, proliferative capacity, various sources for isolation, and differentiation potential into multiple lineages. Clinical studies using human bone marrow-mesenchymal cells as allografts have demonstrated practical use of MSCs for tissue-repair [18, 19]. MSCs can directly or indirectly affect the outcome after transplantation *in vivo* because of their ability to secrete various factors such as angiogenic, anti-apoptotic, proliferation-stimulating factors, and neurotrophic factors [47, 48, 72]. Such utility for MSCs has also been suggested by MSC transplantation into the eyes of experimental glaucoma models followed by subsequent neuroprotective effects on the retinas [73]. MSCs, furthermore, can be genetically modified to express bioactive molecules so they can act as a delivery vehicle for the factors *in vivo*. BDNF-secreting MSCs transplanted into neurodegenerative eyes provided notable preservation of the host retinas morphologically and functionally [74].

Our study indicates that MSCs from Brown Norway rats have a potential to be a cell source for stem cell-based therapies due to their fast and consistent proliferation, and ability for multipotent differentiation. In addition, our MSC line provides an additional choice for rat strain as well as stem cell line, which are important factors to develop strategies for a stem cell-based therapy. More importantly, we provide the first evidence that bone marrow MSCs are capable of promoting neurite outgrowth from adult hippocampal progenitor cells (AHPCs). This would be another intriguing value for MSCs to be utilized for rescuing injured or degenerating nervous systems.

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Figure Legends

Table 1. Antibodies used in this study.

Table 2. Population doubling time (PDT) for MSCs at different passages compared between male- and female-MSCs. To study the growth of BN-MSCs, PDT was calculated at different passages- 1, 4, 9, and 15- for both male and female MSC populations. PDT values, in general, have a range between 18-37 hours. There were no significant differences between

the different passages/or between MSCs, isolated from male versus female Brown Norway rats. (P value > 0.05). (Sample size; N=3)

Table 3. BrdU assay. MSCs were incubated with BrdU (final concentration 5 μM) for 24 hours and immunolabeled with a BrdU antibody to investigate the proliferation rate. At all passages, more than 80% of MSCs were BrdU-positive, except P4 (♂). BrdU percentage of male MSCs at P4 showed a significant difference, compared to that of both male and female MSCs at all the passages examined (P value $<$ 0.05; Sample size N=3).

The results indicate that the majority of the MSCs were in an actively dividing state throughout the passages examined.

Table 4. Percentages of differentiating AHPCs after co-culture with MSCs.

AHPCs were cultured with MSCs for 7 days to study whether co-culture influences the ratio of the differentiation of AHPCs. Three different conditions were tested: 1) AHPC only, 2) NCCC, and 3) CCC. No significant differences between groups were found for the percentages of TuJ1-, RIP-, and BrdU-IR cells (P value $>$ 0.05). Three independent experiments were done for the analysis (N=3). Value= Mean % ± S.E.M. Abbreviations- AHPC: adult hippocampal progenitor cells; NCCC: non-contact co-culture; CCC: contact co-culture.

Fig 1. (A) Phase contrast images of MSCs (male) at initial isolation (Passage 1, P1) and after 10 passages (P10). MSCs isolated from BN-rat bone marrow were cultured as a monolayer on plastic culture plates. Both small, rapidly self-renewing MSCs and larger, more mature MSCs were observed. MSCs had a spindle-shape and fibroblast-like morphology. In later passages, a greater proportion of cells displayed a flattened, fibroblast-like morphology. Scale bar=200 µm.

Fig 2. Characterization of MSCs immunostained with a panel of phenotypic markers.

(A - F) Bone marrow-derived cells stained with a panel of MSC specific -positive antibody markers. No specific staining with MSC negative antibodies (CD11b, CD14, CD44, and CD45) was detected. (CD14- present on leukocytes; CD44- present on leukocytes and endothelial cells; CD45- present on monocytes and macrophages). (K and L) No staining was found with mouse- and rabbit-IgG antibodies, negative controls. Scale bar= 50 µm (A through L).

Fig 3. Differentiation of MSCs- Adipogenesis and osteogenesis.

(A - A') MSCs were cultured in adipogenic induction media for 21 days. Adipocytes differentiated from MSCs were stained with Oil Red O Solution. Lipid droplets in adipocytes were cleary observed. Cell nuclei were stained with Hematoxylin solution. (B - B') For osteogenesis, MSCs were differentiated in osteogenic induction media for 14 days. Osteocytes containing calcium deposits were visualized with Alizarin Red Solution. (Scale bars- A and B: 300 µm; A': 100 µm ; B': 200 µm)

Figure 4. Contact Co-culture (CCC) of AHPCs with MSCs. AHPCs were co-cultured in physical contact with the MSCs. An image shown here is contact co-culture condition, CCC. The AHPCs, which were expressing GFP, could be identified from the non-GFP-expressing MSCs. Image was captured at 3 DIV of co-culture. Scale bar= 50 µm.

Figure 5. Sholl analysis for the quantification of neurite outgrowth of TuJ1-immunoreactive AHPCs.

AHPCs, after 7 days co-culture with MSCs, were immunostained with a neuronal marker (TuJ1). Two representative images from each group are presented, from AHPC only (A, A'), NCCC (B, B'), and CCC (C, C') groups. Sholl analysis was performed to measure the extent of neurite growth and branching from TuJ1-IR cells. Tthree concentric circles (radii 10, 20, and 30 µm from the center of the cell soma) were superimposed onto TuJ1-IR cells. The number of neurite intersections with each concentric circle was counted. AHPCs in both NCCC and CCC conditions displayed increased neurite outgrowth compared to that of AHPC only group.

D. Neurite complexity summary bargraph. Neurite outgrowth of AHPCs after co-culture with MSCs. Neurite outgrowth of neuronal AHPCs (TuJ1-IR) was quantified by performing Sholl analysis. After 7 days co-culture with MSCs, there were significantly increased number of neurite intersections at a radius of 20 µm (AHPC only vs. NCCC and AHPC only vs. CCC). Error bars= S.E.M.; *: P value < 0.05.

Figure 6. Oligodendrocyte differentiation of AHPCs following co-culture with MSCs. AHPCs were co-cultured with MSCs for 7 days and immunostained with a oligodendrocyte marker, RIP. Nuclei were stained with DAPI. AHPCs in both NCCC and CCC conditions showed a more highly branched morphology and in general, a larger area of processes than that of the AHPC only group. Also, in CCC condition, the thickness of primary processes was generally found to be greater than that of other groups (Indicated with an arrow head). Scale bar=50 μm.

Supplementary Fig 1. Characterization of rat bone marrow-derived cells isolated from female (♀) Brown Norway rats immunostained with a panel of MSC positive antibodies.

(A-F) Cells were immunostained with a panel of MSC-positive markers; CD54, CD90, fibronectin, collagen type I, CD29, and CD51. No specific staining was found with MSC-negative markers. Scale bar= 50 μm (Applied from A through F).

Supplementary Fig 2. Endogenous expression of a neural marker

MSCs were immunostained with a neural marker, nestin, without neural induction. About 30 % of MSCs were nestin-IR, showing specific filamentous staining pattern in the cells. Nuclei were stained with DAPI. Scale bar=50 μm.

Supplementary Fig 3. Propidium iodide (PI) staining. To test the viability of MSCs, the cells were incubated with propidium iodide (final concentration 1.5 μM) which identifies dead cells in a population due to the permeability of PI to cells with compromised membrane integrity. (A) No PI-positive cells were found in the population of MSCs maintained in normal culture conditions. (B) PI control condition: MSCs incubated with 70% ethanol prior to PI incubation were all positively labeled by the PI staining. Images shown were merged (DAPI + PI). Repetitive experiments were performed on PI staining. (Scale bar=100 μm; Sample size N=3)

Supplementary Fig 4. Differentiation of MSCs (♀)- Adipogenesis and osteogenesis.

(A - A') MSCs were cultured in adipogenic induction media for 21 days. Adipocytes differentiated from MSCs were stained with Oil Red O Solution. Lipid droplets in adipocytes were cleary observed. Cell nuclei were stained with Hematoxylin solution. (A") Astrocytes served as a negative control for MSC adipogenesis and showed no staining after being subjected to the adipogenic induction conditions. (B - B') For osteogenesis, MSCs were differentiated in osteogenic induction media for 14 days. Osteocytes containing calcium deposits were visualized with Alizarin Red Solution. (B") Astrocytes served as a negative control for MSC osteogenesis and showed no staining after being subjected to the osteogenic induction conditions. Scale bars- A and B: 300 μm; A' - A'': 100 μm ; B' - B'': 200 μm.

Tables and Figures

Table 1. Primary antibodies used in this study

Antibody	Species	Source (clone)	Dilution
MSC markers			
Fibronectin	Rabbit	Millipore	1:1,500
Collagen type I	Rabbit	Millipore	1:500
CD 29 (Integrin beta1)	Rabbit	Millipore	1:500
CD 51 (Integrin alpha V)	Rabbit	Dr. Thomas Joos, NMI, University of Tuebingen	1:200
CD90 (Thy-1.1)	Mouse	Dr. Alan F. Williams, University of Oxford	1:200
CD54 (ICAM-1)	Mouse	Millipore	1:100
CD11b	Mouse	Millipore	1:100
CD45	Mouse	Millipore	1:100
CD14	Mouse	Millipore	1:1,000
CD44 (Hermes-1)	Rat	Developmental Studies Hybridoma Bank (DSHB)	1:200
Mouse IgG	Mouse	Millipore	1:100
Rabbit IgG	Rabbit	Millipore	1:500
Proliferation marker			
BrdU	Rat	DAKO Corp.	1:100
Neural markers			
Nestin	Mouse	DSHB	1:200
III β-tubulin (TuJ1)	Mouse	R&D systems	1:200
MAP2ab	Mouse	Sigma	1:200
GFAP	Mouse	Fisher Scientific	1:500
RIP	Mouse	DSHB	1:1,000

Table 2.

PDT (hrs)		P1	P4	P9	P15
MSC (♀)	Mean	23.74	37.01	25.76	28.73
	± S.E.M.	7.712	4.925	7.072	7.743
MSC (♂)	Mean	17.68	29.67	25.5	23.43
	± S.E.M.	4.137	2.164	2.629	2.563

Table 3.

% BrdU		P1	P4	P9	P15
MSC (♀)	Mean	93	88.67	83.67	83.33
	± S.E.M.	2.517	3.844	7.172	4.096
MSC (♂)	Mean	89.33	54.76	84.33	85.33
	± S.E.M.	4.372	5.219	1.453	5.783

Table 4. Differentiation of AHPCs after Co-culture with MSCs

	AHPC	NCCC	CCC
TuJ1	21.46	23.11	19.74
	± 8.765	± 4.473	± 6.918
RIP	59.15	29.45	54.61
	± 13.04	± 8.102	± 7.98
BrdU	36.89	29.76	38.23
	± 10.22	± 7.648	± 10.94

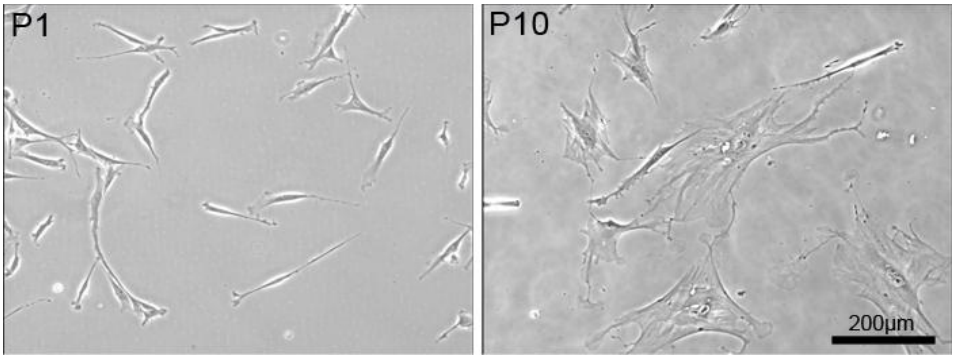


Fig 1. Morphology of MSCs

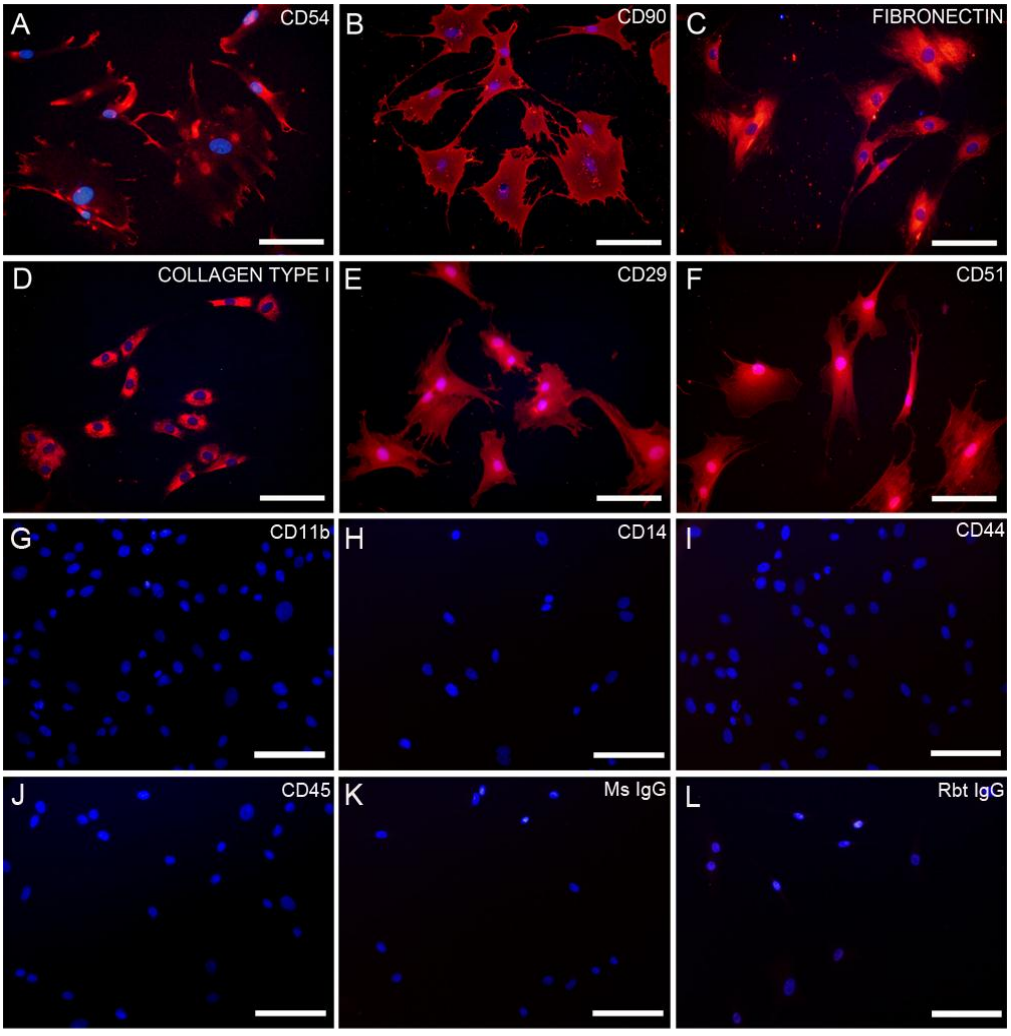


Fig 2. Characterization of MSCs

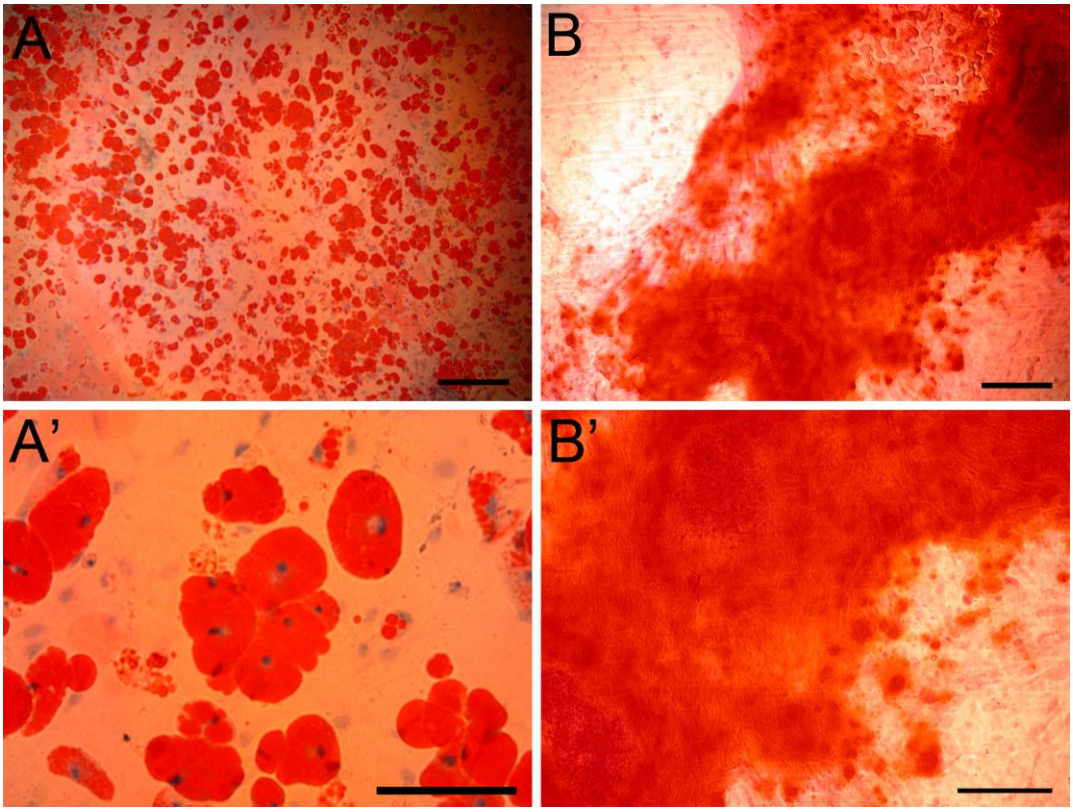


Fig 3. Differentiation of MSCs- Adipogenesis and osteogenesis

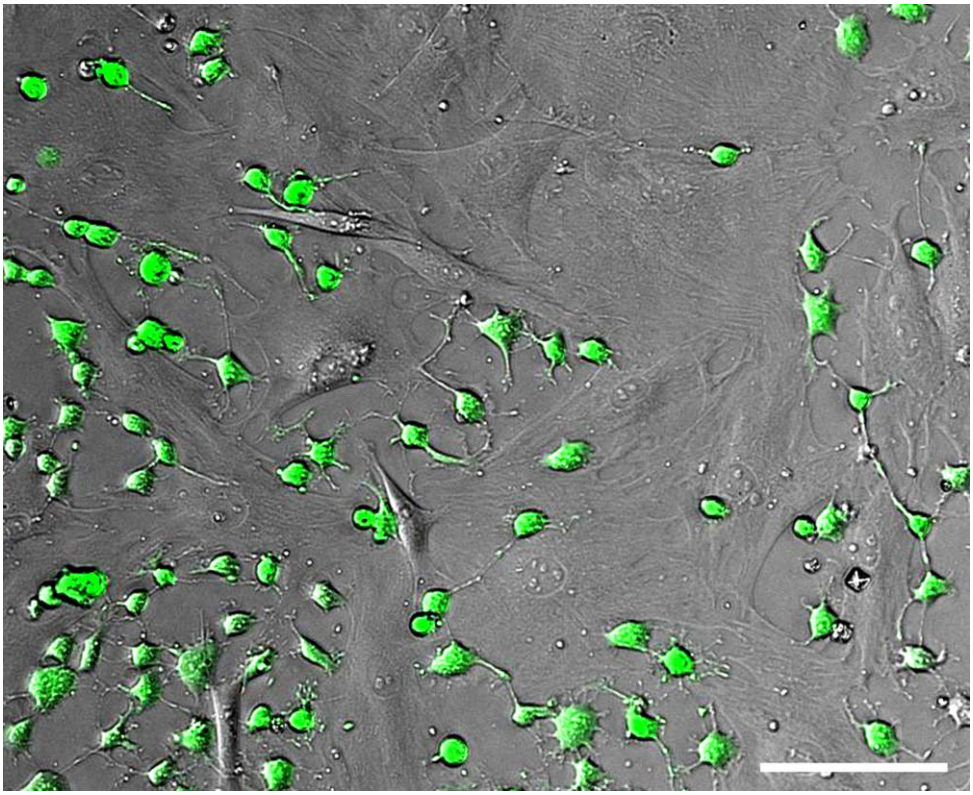
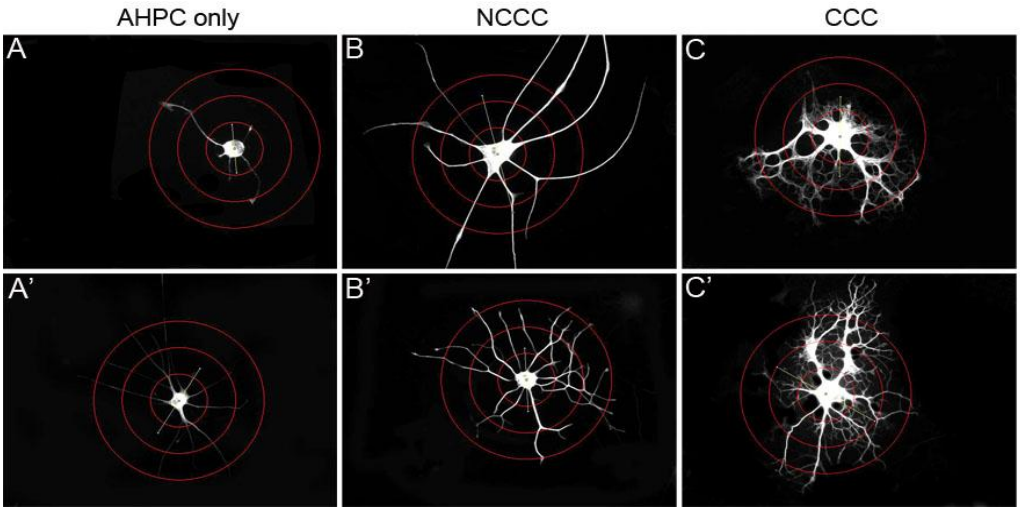


Figure 4. Contact Co-culture (CCC) of AHPCs with MSCs.



(D)

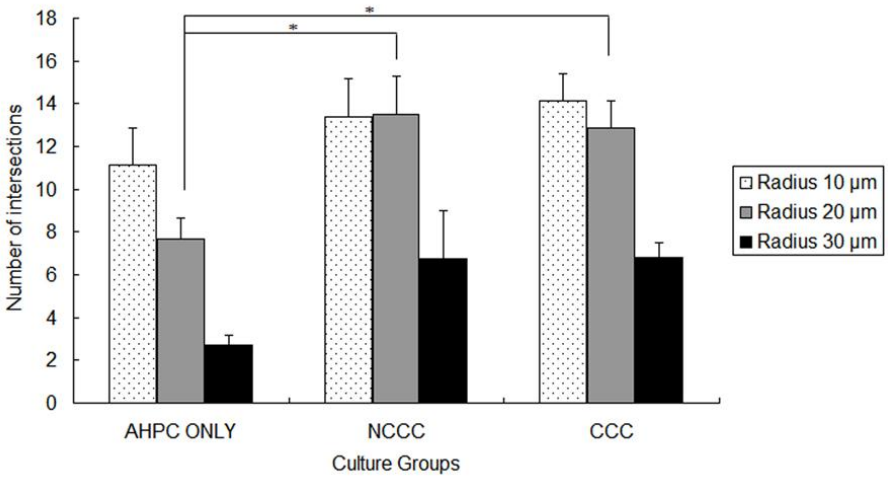


Figure 5. Sholl analysis for the quantification of neurite outgrowth of the AHPCs.

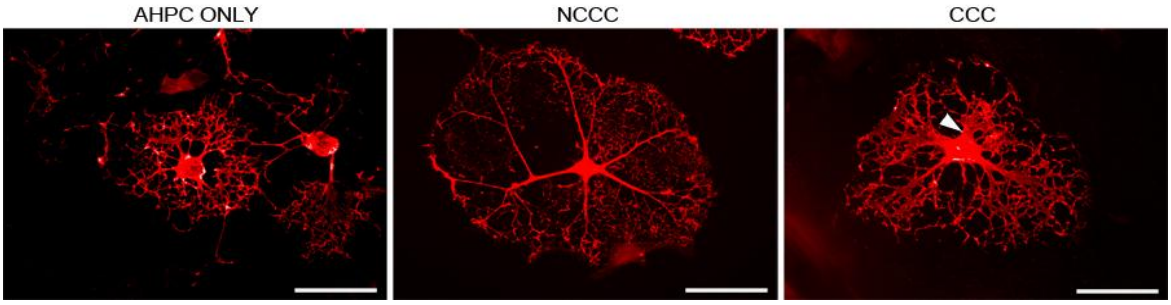
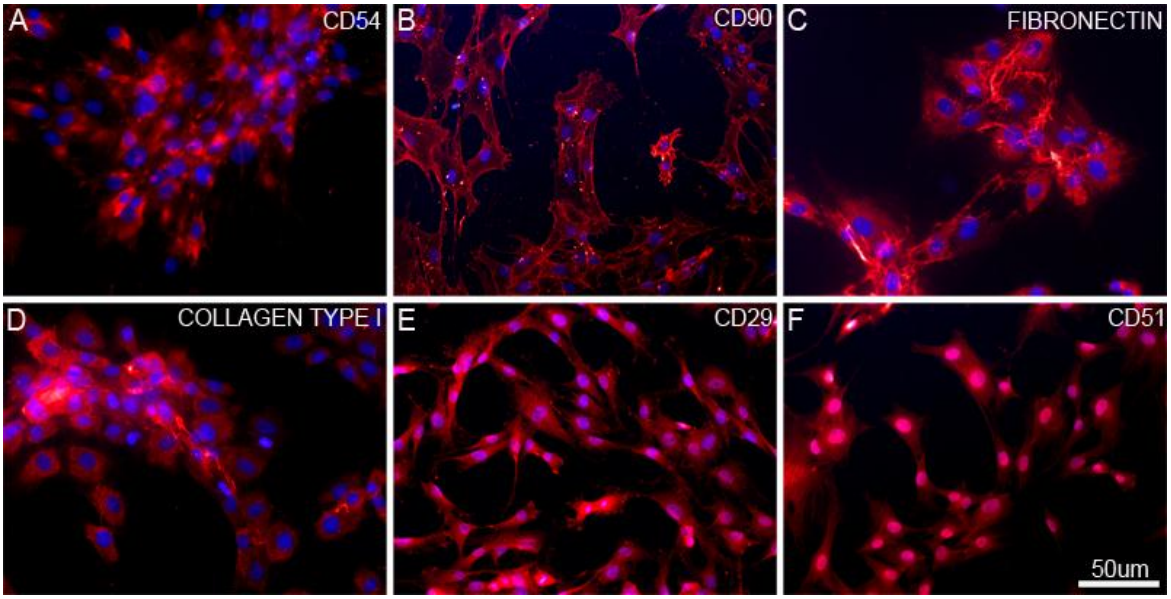
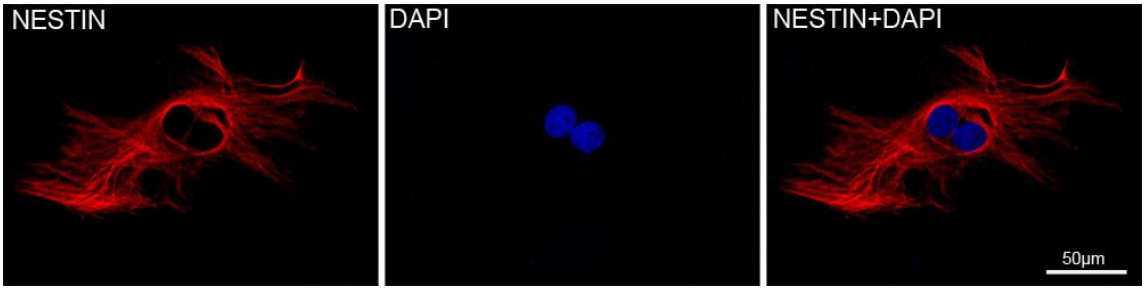


Figure 6. Oligodendrocyte differentiation of AHPCs following co-culture with MSCs.

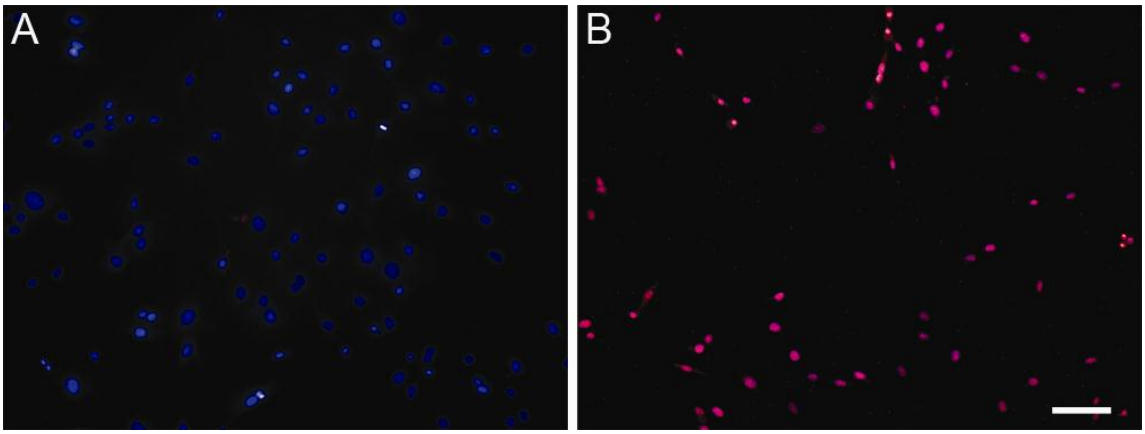
Supplementary materials (Data of ♀ BN-MSCs)



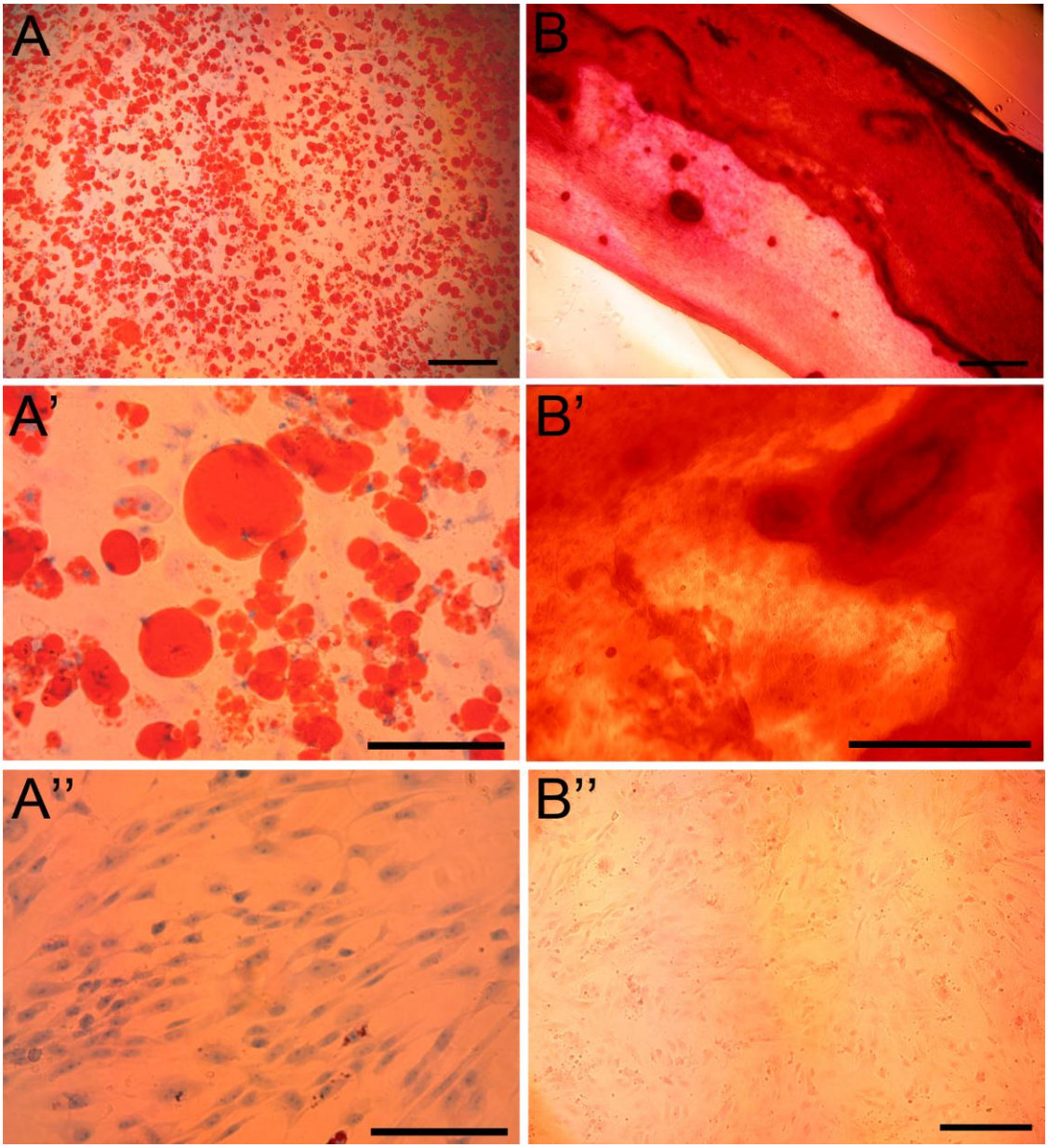
Supplementary Fig 1. Characterization of MSCs



Supplementary Fig 2. Endogenous expression of neural marker



Supplementary Fig 3. PI staining on MSCs



Supplementary Fig 4. Differentiation of MSCs- Adipogenesis and osteogenesis

CHAPTER 3: ENGINEERING MOUSE MESENCHYMAL STEM CELLS DERIVED FROM BONE MARROW AND *IN VITRO* CHARACTERIZATION

This chapter is a part of the paper to be submitted to Stem Cell Research.

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Abstract

The purpose of this study was to develop cellular vehicles to deliver neurotrophic factors as a part of neuroprotective strategies for rescuing neurodegenerative retina. In the U.S., millions of people suffer from ocular diseases, which eventually can lead to vision loss. Glaucoma, the second leading cause of blindness in the world, is a hereditary ocular disease which afflicts about 60 million individuals [1]. Currently, there are no remedies to cure glaucoma. As a potential strategy to rescue degenerating retinas, stem cells have been investigated extensively and the effects of morphological neuroprotection or functional recovery have been reported [2-5]. Mesenchymal stem cells (MSCs) are self-renewing, multipotent stromal cells and can be isolated from a variety of regions in the body. Bone

marrow-MSCs are relatively easily isolated and have been used in numerous studies as a potential candidate to develop therapeutic strategies for various diseases [6-22]. More importantly, bone marrow-MSCs are a potential candidate for autologous transplantation, which avoids immune responses in the host. In this study we infected mouse bone marrow-derived MSCs with lentiviral vectors, which encode brain-derived neurotrophic factor (BDNF)/or glial cell-derived neurotrophic factor (GDNF) together with green fluorescent protein (GFP). Using fluorescence-activated cell sorting (FACS), enriched populations of the different GFP-expressing cells were collected. Our data showed MSCs infected with lentiviral vectors noticeably increased the expression of BDNF and/or GDNF protein in the cells. In addition, the amount of the secreted neurotrophic factors was highly increased and these were bioactive, as demonstrated by ELISA and an *in vitro* bioassay, respectively.

These engineered MSCs were transplanted into mouse eyes of glaucoma models as potential neuroprotectors *in vivo*. The outcome is discussed in the next research chapters.

Introduction

Bone marrow-derived mesenchymal stem cells (MSCs) are a potential source for stem cell-based therapies, since they have several advantages, including 1) easy isolation and culture, 2) significant plasticity, 3) no ethical concerns, 4) capability to survive and migrate *in vivo* after transplantation [23, 24], 5) potential autologous transplant, and 6) availability of engineering to produce a cellular vehicle for long-term neurotrophic factor delivery. The axoplasmic flow of retinal ganglion cells is disturbed in the condition of ocular hypertension and, thus, retrograde transport of neurotrophic factors and the receptors are interrupted [25-

28]. Therefore, many studies have been performed to deliver the interested genes or proteins to the target regions to prevent neuronal death from the deprivation of trophic factors. Injection of purified proteins, such as ciliary neurotrophic factor (CNTF) [29], Adeno-associated viruses (AAVs) encoding trophic factors [30], or microspheres releasing GDNF [31, 32] into the eyes of animal models, have shown neuroprotective effects on the degenerating RGCs to some extent. However, these methods do not provide consistent and long-term support of trophic factors to the target region of interest.

Taking into consideration the numerous advantages of MSCs, it is possible that delivery of neurotrophic factors via MSCs may be a useful strategy to overcome such limitations of short-term effects or an uncontrolled release of proteins *in vivo*. Utilizing MSCs, as a cellular vehicle to deliver neurotrophic factors, has been successful to rescue injured or degenerating retinal cells *in vivo* [4, 5].

Engineering MSCs *ex vivo* is another powerful strategy for the delivery of the genes and gene products of interest. Viral vectors can be used as a potent tool for delivering genes to engineer MSCs. In this study, lentiviral vectors (LVs) were used to genetically engineer MSCs. Lentiviral vectors (LV) are a potent and efficient construct to deliver genes into non-dividing and dividing cells. In addition, lentiviral constructs are a self-inactivating vector, possess the capability to integrate into the genome of host cells, and can result in sustained expression of the transgene [33-35]. Lentiviral vectors have been studied for use in gene therapy *in vivo* as well and have shown success on efficient transfer of genes and the protection of degenerating retinas [36-44].

However, concerns with these vector applications exist, due to the low efficiency of viral infection to mature retinal neurons *in vivo* [45] and transient toxicity on the retinas [46]

when used directly into the eyes. Thus, we utilize lentiviral vectors to infect stem cells *ex vivo* for cell-based therapy. These constructs are a very powerful and desirable vehicle to deliver genes into the cells, as they are highly efficient for cell infection and can be manufactured on a large scale for use in therapeutic treatments [47].

In this study we have employed lentiviral vectors to engineer bone marrow derived-MSCs as cellular delivery vehicles for BDNF and GDNF. Our results demonstrate lentiviral constructs infect MSCs efficiently and induce significant increase of BDNF and GDNF expression in the MSCs. In addition, the levels of secreted BDNF and GDNF are significantly greater than control MSCs, and the secreted neurotrophic factors are bioactive.

These engineered MSCs may have significance in therapeutic applications to serve as transplants to deliver neurotrophic factors for neurodegenerative diseases. Our strategy is to use engineered MSCs as a cellular vehicle of neurotrophic factor delivery for the glaucomatous eye. The MSCs are transplanted into glaucomatous eyes in animal models and can survive for a long-term period, releasing bioactive neurotrophic factors.

Materials and Methods

Mouse mesenchymal stem cells (MSCs)

Mouse MSCs isolated from the bone marrow of adult C57BL/6 mice were obtained from Tulane Center for Gene Therapy (New Orleans, LA) and maintained as an adherent cell line in Iscove's Modified Dulbecco's Medium (IMDM; 12440–046; Invitrogen, Carlsbad, CA) containing 10% hybridoma-qualified FBS (Cat #-SH30396.03, Hyclone, Logan, UT), 10% equine serum (Cat #- SH3007403, Hyclone, Logan, UT), 2 mM L-glutamine, and

10,000 U/mL penicillin, 10,000 µg/mL streptomycin. When cultures reached 70–80% confluence, MSCs were gently detached from the flask using a 0.05% trypsin and 0.1% EDTA solution (Invitrogen) and were pelleted at 500 rpm for 5 minutes. MSCs were subsequently plated into T75 flasks at low confluence. Cultures were maintained by supplementing the flask with fresh medium every other day.

Engineering MSCs *Ex Vivo* with lentiviral vectors

MSCs were infected using lentiviral vectors to engineer them to secrete brain-derived neurotrophic factor (BDNF; human cDNA) and glial cell-derived neurotrophic factor (GDNF; human cDNA), as previously reported [48]. Briefly, MSCs were plated in six-well plates at a density of 10,000 cells per well and were allowed to adhere for a day. After adhering to the plate, the growth medium in each well was replaced with IMDM, containing 2% FBS and 12 µg/mL Sequa-brene (152667; Sigma-Aldrich, St. Louis, MO). Each lentiviral vector's encoding BDNF (LV-BDNF; CMV-BDNF-IRES-GFP), GDNF (LV-GDNF; CMV-GDNF-IRES-GFP), and green fluorescent protein (GFP, LV-GFP; CMV-GFP) were added separately to MSCs at a multiplicity of infection (MOI) of 1,000 for each vector. Media containing viral particles were removed after 8 hours of incubation and fresh growth medium was added. When confluence reached 70–80%, MSCs were transferred to a flask for subsequent maintenance. Subcultures were performed as described above. MSCs, that expressed strong green fluorescence, were collected by fluorescence-activated cell sorting (FACS). To sort MSCs by FACS, MSCs (BDNF- and GDNF-MSCs) were collected in separate 15 ml tubes. Each sample tube contained 3 to 5 million MSCs in 0.5 ml culture

media. Then, MSCs with strong green fluorescence were collected in separate catch tubes containing 1 ml FBS. Additionally, GDNF/BDNF-MSCs were generated by infecting GDNF-MSCs, which were previously sorted by flow cytometry, with the LV-BDNF vector at a MOI of 1,000.

ELISA of BDNF and GDNF production

Enzyme-linked immunosorbent assay (ELISA) was performed to quantify the amount of BDNF and GDNF released from GFP-MSCs, BDNF-MSCs, GDNF-MSCs, and GDNF/BDNF-MSCs. BDNF- and GDNF-assays (Emax Immunoassay System, G7611 and G7621, respectively; Promega Corporation, Madison, WI) were used to detect BDNF and GDNF, respectively, in media conditioned for 24 hours by each group of MSCs. Initially, 250,000 cells were plated into a T25 flask and allowed to adhere for a day. Media were then replaced with fresh growth media, and cells were grown for an additional 24 hours. Conditioned media (CM) were collected, filtered using sterile 0.2 μm syringe filters, and frozen at -20°C for subsequent analysis. For BDNF assay, anti-BDNF monoclonal antibody in carbonate buffer was used to coat the wells of a 96-well plate overnight at 4°C. These wells were incubated with blocking and sample buffer for 1 hour at room temperature. Purified BDNF was used to generate the standard curve (0–500 pg/well). Samples from CM were prepared and incubated in wells on a shaker for 2 hours. Wells were subsequently washed. An anti-BDNF polyclonal antibody was added to the wells and plates were incubated again on a shaker for 2 hours. An anti-IgY horseradish peroxidase–conjugated antibody was incubated in the wells on a shaker for 1 hour at room temperature and

subsequently washed. Chromagen substrate 100 µL (TMB-One solution; Promega) was added to each well and reacted for 10 minutes at room temperature. Reactions were stopped by adding 100 µL of 1 N HCl per well.

For GDNF assay, an anti-GDNF monoclonal antibody in a carbonate buffer was utilized to coat the wells of a 96-well plate overnight at 4°C. Wells were incubated with a block and sample buffer for 1 hour at room temperature. Purified GDNF was used to generate the standard curves (0–1,000 pg/well). Samples from CM were prepared and incubated in wells on a shaker for 6 hours. Wells were subsequently washed. An anti-GDNF polyclonal antibody was added to the wells and plates were incubated overnight at 4°C. An anti-Chicken IgY, horseradish peroxidase–conjugated antibody was incubated in the wells on a shaker for 2 hours at room temperature and subsequently washed. Chromagen substrate 100 µL (TMB-One solution; Promega) was added to each well and reacted for 10 minutes at room temperature. Reactions were stopped by adding 100 µL of 1 N HCl per well. All plates were read using a microplate reader at 450 nm.

Bioactivity assay of neurotrophic factors

Bioactivity for BDNF and GDNF released from the MSCs was examined using an *in vitro* assay. For this bioassay, PC12-TrkB cells (a generous gift from Dr. Chao, NYU) and PC12 cells (ATCC, Manassas, VA) were used to test for neurite outgrowth promoting activity [79, 80] of the MSC-derived BDNF and GDNF, respectively. Cells were plated onto a laminin (10 µg/ml)-coated surface of a 24-well culture plate and allowed to attach to the surface for a day. The next day, CMs were added into each well and cultures were

maintained in a mixed medium consisting of PC12-trkB cell/or PC12 cell growth media in a 1:1 mixture with MSC-CM. The following conditions were tested: five different groups of CMs collected from 1) wild-type (Wt) MSCs, 2) GFP-MSCs, 3) BDNF-GFP-, 4) GDNF-GFP-, 5) GDNF/BDNF-MSCs, and 6) fresh maintenance medium (MM), and 7) 50 ng/ml NGF (nerve growth factor), 8) 50 ng/ml recombinant human BDNF (rhBDNF, 450-02; PeproTech, Rocky Hill, NJ), or 9) 50 ng/ml recombinant human GDNF (rhGDNF, Cat #-450-10)-containing MSC media as positive controls. Cells were cultured up to 6 days for each condition. Phase contrast images were taken to perform a semi-quantitative analysis of neurite outgrowth. The level of neurite outgrowth was scored from 0 to 4, with 0 representing little neurite growth in terms of length and density, and 4 representing long, dense neurite outgrowth (Fig. 3).

Immunocytochemistry

For immunolabeling, MSCs were fixed in 4% paraformaldehyde in 0.1 M PO_4 buffer, pH 7.4. Fixed cells were incubated in blocking solution containing 5% normal donkey serum, 0.4% bovine serum albumin (BSA; Sigma), and 0.2% Triton X-100 (Fisher Scientific), followed by incubation with primary antibodies overnight at 4°C. Primary antibodies used were anti-BDNF (1:200, Santa Cruz Biotechnology, Dallas, TX), anti-GDNF (1:200, Santa Cruz Biotechnology, Dallas, TX). After rinsing in PBS, cells were incubated in the secondary antibodies conjugated to Cy3 diluted at 1:500 (Jackson ImmunoResearch, West Grove, PA). Cell nuclei were stained with 4', 6-diamidino-2-phenylindole, dilactate (DAPI), diluted at 1:1,000 in PBS and applied for 30 minutes. Preparations were rinsed and then mounted onto

microscope slides using an antifade mounting medium (Gel Mount; Biomeda Corp., Foster City, CA). Negative controls were performed in parallel by omission of the primary antibodies. No antibody labeling was observed in the controls.

Quantitative analysis of neurotrophic factor expression on the MSCs

To analyze the expression level of BDNF and GDNF on the MSCs, a pixel count was performed on the staining of BDNF and GDNF using ImageJ software. Images were taken with 630X magnification on a fluorescence microscope and more than 40 cells per MSC group were anayzed for pixel count. The threshold for the minimum pixel intensity value was set so the program would recognize a baseline where no background fluorescence was observed in the image. Then, the integrated intensity value was measured for BDNF and GDNF staining. Lastly, the intensity value was divided by unit area for each cell so that the values from different sized cells can be compared.

Imaging and statistics

Images of MSCs labeled with the antibodies were captured using an upright fluorescence microscope (Nikon Microphot FXA) equipped with a Retiga 2000R digital camera controlled by QCapture software (QImaging, Surrey, British Columbia, Canada). Figure plates were prepared using Photoshop CS2. Data were reported as means ± S.E.M. Statistical analysis was performed using GraphPad PRISM (ver. 3.0). All tests were two-tailed tests and P values less than an alpha of 0.05 were considered significantly different.

Results

Increased expression of neurotrophic factors in the MSCs

We sought to generate different subpopulations of MSCs that release BDNF, GDNF, or both BDNF and GDNF together with GFP. Cultured MSCs were infected with three different LV-constructs at a MOI of 1,000 for each vector, and four types of LV-MSCs were engineered; GFP-, BDNF-, GDNF-, and GDNF/BDNF-MSCs. Infected cells, concurrently expressing green fluorescent protein, were visualized under a fluorescent microscope to assess the efficiency of the viral infection. Infection with GFP-LVs showed 100% efficiency and all the GFP-MSCs displayed a strong green fluorescence (Figure 1). Infection efficiencies for the BDNF- and GDNF-MSCs was between approximately 50 and 80%, based on GFP-expression. To enrich for the infected cells (based on GFP-expression), these MSC populations were subjected to FACS analysis. A population of MSCs expressing both GDNF and BDNF were produced by infecting the GDNF-MSCs (previously enriched by FACS) with the BDNF lentiviral vector. After FACS, all MSC populations were expanded further.

The expression of the neurotrophic factors in the MSCs was examined by immunocytochemistry, using specific antibodies directed against BDNF and GDNF (Figure 1). A stronger immunoreactivity (IR) of BDNF was observed on the BDNF- and GDNF/BDNF-MSCs, compared to that for GDNF- and GFP-MSCs. Robust BDNF expression was observed on the cell body, including the nucleus. No specific IR was found on the negative control stain, done by omitting the primary antibodies (Figure 1A).

Immunoreactivity for GDNF expression was greatest on the GDNF- and GDNF/BDNF-MSCs, compared to that for GFP- and BDNF-MSCs. A notably stronger IR

was observed on the MSCs infected with GDNF-LVs. The cellular location of GDNF was present in the cell body, but not in the nucleus (Figure 1B).

The levels of neurotrophic factor expression on the MSCs were quantified by analyzing the number of pixels per unit area (Figure 2). In accordance with Figure 1, both BDNF- and GDNF/BDNF-MSCs showed prominently increased expression of BDNF than for GDNF-MSCs. Also, both GDNF- and GDNF/BDNF-MSCs showed an elevated level of GDNF expression, compared to BDNF- and GFP-MSCs. Moreover, GDNF/BDNF-MSCs expressed increased levels of GDNF as well as BDNF. Additional experiments are required to conclude statistical significance of the values.

Enhanced secretion of neurotrophic factors from the MSCs

To examined whether engineered MSCs were capable of secreting BDNF and GDNF, ELISA analyses were conducted on the media conditioned by the engineered MSCs (Table 1). No detectable BDNF and GDNF were found from the conditioned media (CM) from the control, GFP-MSCs. However, BDNF-MSCs released an increased amount of BDNF (5.9 ng/10^6 cells/24 hrs) while GDNF was not detected. An elevated amount of GDNF (34.1 ng/10^6 cells/24 hrs) was detected in the GDNF-MSC CM, with no detectable BDNF. GDNF/BDNF-MSCs secreted comparable amounts of BDNF (3.2 ng/10^6 cells/24 hrs) and GDNF (33.8 ng/10^6 cells/24 hrs) to that of BDNF- and GDNF-MSCs, respectively. Together these results demonstrate that the lentiviral transduced MSCs were capable of producing and secreting the respective neurotrophic factor.

Bioactivity of neurotrophic factors released from the MSCs

An *in vitro* assay was implemented to investigate the bioactivity of the neurotrophic factors released from the engineered MSCs. PC12 cell lines were cultured in media conditioned by the different MSC populations to determine the effect of MSC secreted neurotrophic factors on neurite outgrowth. Bioassays were performed on a laminin (10 µg/ml)-coated surface of a 24-well culture plate. 500 µl of media, 1:1 mixture of PC12 cell culture media with MSC-CM, were added into each test wells. A total of nine different conditions were tested: MSC CMs collected from 1) Wt-, 2) GFP-, 3) BDNF-, 4) GDNF-, and 5) GDNF/BDNF-MSCs, and also controls, including 6) fresh maintenance medium (MM), 7) 50 ng/ml NGF, 8) 50 ng/ml recombinant human BDNF (rhBDNF), and 9) 50 ng/ml recombinant human GDNF (rhGDNF). Wt-MSCs were used as a cell control and they were not infected by lentiviral vectors. By culturing PC12-TrkB cells in MSC CMs (Figure 4A), the bioactivity for BDNF was assessed. At 4 days *in vitro* (DIV), the neurite outgrowth of PC12-TrkB cells had significantly increased in BDNF- and GDNF/BDNF-MSC CMs, compared to those for the Wt-, GFP-, and GDNF-MSC CMs and control MM conditions. The growth of neurites in BDNF- and GDNF/BDNF-MSC CMs was comparable to that for the positive control—50 ng/ml rhBDNF.

Bioactive GDNF was revealed from the culture of PC12 cells in MSC CMs (Figure 4B). At 6 DIV of culture, a significantly increased level of neurite outgrowth of PC12 cells was measured in GDNF- and GDNF/BDNF-MSC CMs, compared to that in the Wt-, GFP-, and BDNF-MSC CMs and MM. The neurite outgrowth enhanced in the GDNF- and GDNF/BDNF-MSC CMs was comparable to that for the positive control—rhGDNF 50 ng/ml. Statistical significance for the differences is shown on Table 2. These results

demonstrate that both BDNF and GDNF released from genetically engineered MSCs are bioactive *in vitro*. Also, GDNF/BDNF-MSCs secrete comparable amounts of GDNF and BDNF to enhance neurite outgrowth, compared to that of GDNF- and BDNF-MSCs, respectively.

Additionally, we have confirmed stable expression and release of neurotrophic factors from engineered MSCs, which have been subcultured for an extended period of time. Also, repetitive freezing-thaw procedures have not affected the capability of MSCs to release neurotrophic factors.

Discussion

We have demonstrated the lentiviral infection on the MSCs is a competent method to deliver genes of interest. Genetic engineering using lentiviruses is a very powerful and efficient strategy to deliver genes into cells, as it allows a stable and long-term expression of the genes [33, 38, 49]. Lentiviral vectors are capable of delivering genes into non-dividing cells [34, 50-52] as well as stem cells [53-55]. We used bicistronic lentiviruses, which allowed the MSCs to concurrently express BDNF/or GDNF with GFP. Thus, the engineered MSCs can be visualized by expression of green fluorescent protein and easily detectable *in vivo* after transplant.

Following the successful delivery of genes, we demonstrate the expression of the neurotrophic factor proteins are elevated in the MSCs. Immunostaining revealed that BDNF-MSCs expressed an increased level of BDNF protein, unlike GFP- and GDNF-MSCs. GDNF-MSCs showed elevated expression of GDNF protein, unlike GFP- and BDNF-MSCs.

Double-infection with GDNF- and BDNF-LVs also resulted in an over-expression of both GDNF and BDNF proteins in the MSCs. Release of GDNF and BDNF from the MSCs has a significance as that may have implications in therapeutic strategy to treat neurodegenerative diseases, including glaucoma. Previous *in vitro* studies have demonstrated neuroprotective effects of BDNF and GDNF on neuronal cells, including RGCs [56-61]. These effects have also been shown in animal models of retinal disease or injury [5, 30, 62-65] as well as brain injury [66].

The co-expression of both GDNF and BDNF in the MSCs is a novel aspect to our strategy. Synergistic or combined effects of BDNF and GDNF have been reported for neuroprotection and differentiation for neurons [67-70]. Previous studies have suggested that BDNF and GDNF act in a distinct mode when they are combined for treatment of motorneurons as they show synergistic or combined effects on neuronal differentiation [67] and neuroprotection [68]. In addition, co-administration of BDNF and GDNF exerts additive effects on RGC survival in a rat model of optic nerve transaction [71].

BDNF has been shown to activate MAPKinase, Ras/Raf/MEK/ERK, and PI3K/Akt/mTOR pathways, which promote cell survival and growth [72-74]. BDNF also binds to p75NT receptor and results in apoptosis via the activity of Jun N-terminal kinase (JNK) [72]. GDNF has been known to activate not only PI3K and MAPkinase pathways, but also PLC-ϒ and Src-family kinase signaling [75]. A study also reported a temporal discrepancy on the phosphorylation of MAPKinase by GDNF and BDNF. GDNF induces a transient phosphorylation, while BDNF results in a long lasting MAPK phosphorylation [74] Thus, we speculate BDNF and GDNF work independently to activate neuronal survival pathways and enhance RGC survival.

In addition to the cellular expression for BDNF and GDNF proteins, we demonstrate the secretion of the neurotrophic factors from the engineered MSCs increased. Slightly higher levels of BDNF-IR on the GFP-MSCs were found, when comparing with ELISA, which had no detectable BDNF (Table 1). This possibly indicates either BDNF protein was produced in the cells, but not secreted, or crossover interference of a strong green fluorescence occurs in the cells. It has been shown that MSCs endogenously express different types of trophic factors, including BDNF, GDNF, NGF, CNTF, neurotrophic -3 (NT-3) [76, 77] as well as immunosuppressive, angiogenic, and proliferation-stimulating factors [78]. A previous study demonstrated that MSCs are capable of secreting BDNF, along with a significant increase of BDNF secretion from the MSCs following BDNF gene delivery [5]. It is possible that the low sensitivity of ELISA kits we utilized may result in a lack of detectable BDNF and GDNF from CM for our GFP-MSCs. Additional studies, such as Western blotting, may also be useful to investigate neurotrophic factor expression in the MSCs, as well as the amount of neurotrophic factor secretion from the MSCs.

We demonstrated the engineered MSCs possess the capability of hyper-secretion of BDNF and GDNF, which are bioactive. These results suggest MSCs are a potential delivery vehicle, which provides neuroprotection to degenerating or damaged cells by secreting neurotrophic factors. Also, MSCs are a robust population, grow fast, and avoid ethical issues related to human embryonic and fetal stem cell isolation. In addition, MSCs have the potency for use in clinical therapy, since they are a useful resource for autologous transplant and can be easily isolated from the body. Also, they have immunomodulatory functions.

I discuss, in the next chapters, *in vivo* investigations of this cellular vehicle system to study the effects of BDNF and GDNF delivery in the experimental glaucomatous models.

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Figure Legends

Table 1. *In vitro* quantification of BDNF and GDNF released from engineered MSCs. ELISA was performed, using conditioned media (CM) collected from each group of MSCs. No BDNF and GDNF were detectable from the control GFP-MSCs, while BDNF-, GDNF-, and GDNF/BDNF-MSCs secreted elevated amounts of BDNF and/or GDNF. (Abbreviation; N.D.: not detectable)

Table 2. Statistical analysis of bioassays. One-way ANOVA with Tukey's multiple comparisons test was used. $P < 0.05$ was considered significant. **** = 0.0001.

Figure 1. *In vitro* expression of BDNF and GDNF on the MSCs after lentiviral infection. To engineer MSCs to over-express BDNF and/or GDNF, wild-type MSCs were infected with lentiviral vectors (LV): GFP-, BDNF/GFP-, or GDNF/GFP-LVs. Infected cells concurrently expressed green fluorescent protein, which can be visualized under a fluorescent microscope. Subsequently, GDNF-MSCs were infected with BDNF-LVs to generate GDNF/BDNF-MSCs. (A) BDNF-MSCs showed stronger immunoreactivity (IR) against BDNF than for GFP- and GDNF-MSCs, and (B) GDNF-MSCs showed a brighter immunoreactivity than for GFP- and BDNF-MSCs. No specific IR was found on the negative control stain, done by omitting the primary antibodies. Scale bars are 50 microns, and all images were taken with the same magnification.

Figure 2**.** Quantification of BDNF and GDNF expression on the MSCs *in vitro.*

MSCs stained against BDNF or GDNF proteins were quantified by measuring the number of pixels per unit area (2000 pixels) using ImageJ. BDNF- and GDNF-MSCs showed a notably increased expression of BDNF and GDNF, respectively. Also, both BDNF and GDNF were highly increased on GDNF/BDNF-MSCs. Error bars are standard deviation. N=1; Number of cells analyzed: > 40 cells per MSC group.

Figure 3. Representative images of bioassay. A semi-quantitative analysis was performed to assess neurite outgrowth from PC12 cells.

(A) BDNF bioassay. PC12-TrkB cells were cultured in MSC CMs to test the bioactivity of BDNF released from MSCs. At 4 DIV, the neurite outgrowth was significantly increased in both BDNF- and GDNF/BDNF-MSC CMs, compared to those for the Wt- and GFP-MSC CMs and control MM conditions. The growth of neurites in the BDNF- and GDNF/BDNF-MSC CMs was comparable to those for the positive controls—50 ng/ml rhBDNF and 50 ng/ml NGF.

(B) GDNF Bioassay. Bioactive GDNF was tested using PC12 cells by culturing in MSC CMs. At 6 DIV of culture, a significantly increased level of neurite outgrowth was observed in both GDNF- and GDNF/BDNF-MSC CMs, compared to those in the Wt- and GFP-MSC CMs, and MM. The growth of neurites in the GDNF- and GDNF/BDNF-MSC CMs was comparable to those for the positive controls—NGF 50 ng/ml and rhGDNF 50 ng/ml. Scale bars = 200 μm.

Figure 4**.** *In vitro* assessment of bioactivity of BDNF and GDNF. The level of neurite outgrowth was scored from 0 to 4, with 0 representing no or little neurite growth in terms of length and density and 4 representing long, dense neurite outgrowth.

(A) Bioactivity of BDNF released from BDNF- and GDNF/BDNF-MSCs was tested using PC12-TrkB cells. At 4 DIV culture, significantly higher levels of neurite outgrowth were measured from both BDNF- and BDNF/GDNF MSC-CMs, compared to those for the control Wt- and GFP-MSC CMs and MM groups.

(B) Bioactivity of GDNF released from GDNF- and GDNF/BDNF-MSCs was tested using PC12 cells. At 6 DIV, significantly increased level of neurite outgrowth of PC12 cells was shown in both GDNF- and GDNF/BDNF-MSC CMs, compared to those for the control groups—Wt- and GFP-MSC CMs and MM.

NGF was used as one of the positive controls for neurite outgrowth of PC12 cell lines. Error bars are standard error. (N = 3)

Tables and Figures

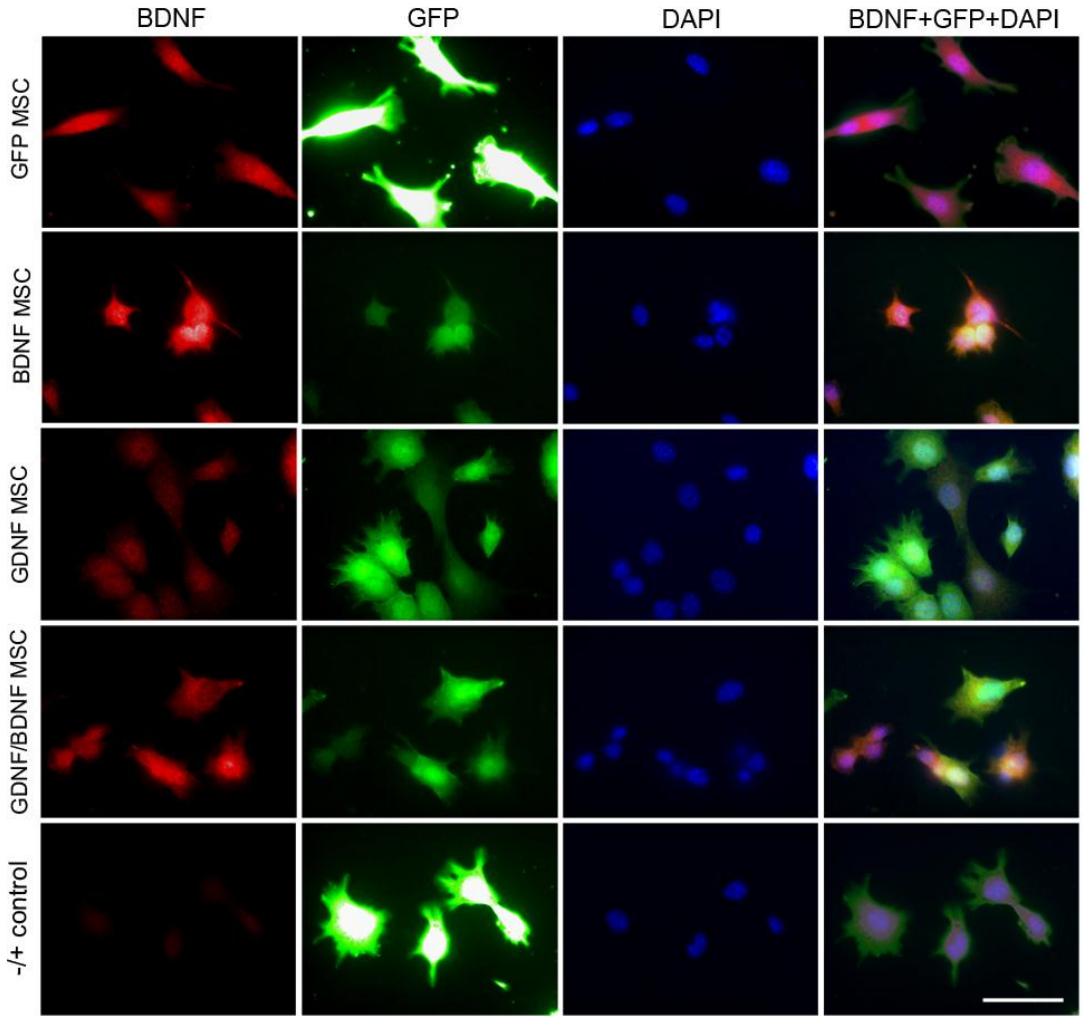
Table 1. *In vitro* quantification of BDNF and GDNF released from engineered MSCs.

MSC Groups	**BDNF**	**GDNF**
GFP MSC	N.D.	N.D.
BDNF MSC	0.59	N.D.
GDNF MSC	N.D.	3.41
GDNF/BDNF MSC	0.32	3.38

(ng/100,000 cells/24 hrs)

Table 2. Statistical analysis of bioassays.

GDNF bioassay	Significance	**BDNF bioassay**	Significance
MM vs. GDNF-MSC	***	MM vs. BDNF-MSC	****
MM vs. GDNF/BDNF-MSC	*	MM vs. GDNF/BDNF-MSC	****
MM vs. NGF	****	MM vs. NGF	****
MM vs. rhGDNF	**	MM vs. rhBDNF	****
Wt-MSC vs. GDNF-MSC	***	Wt-MSC vs. BDNF-MSC	****
Wt-MSC vs. GDNF/BDNF-MSC	*	Wt-MSC vs. GDNF/BDNF-MSC	****
Wt-MSC vs. NGF	****	Wt-MSC vs. NGF	****
Wt-MSC vs. rhGDNF	**	Wt- MSC vs. rhBDNF	****
GFP-MSC vs. GDNF-MSC	***	GFP-MSC vs. BDNF-MSC	****
GFP-MSC vs. GDNF/BDNF-MSC	**	GFP-MSC vs. GDNF/BDNF-MSC	****
GFP-MSC vs. NGF	****	GFP-MSC vs. NGF	****
GFP-MSC vs. rhGDNF	**	GFP-MSC vs. rhBDNF	****
BDNF-MSC vs. GDNF-MSC	***	BDNF- MSC vs. GDNF-MSC	****
BDNF-MSC vs. GDNF/BDNF-MSC	**	BDNF-MSC vs. NGF	*
BDNF-MSC vs. NGF	****	BDNF-MSC vs. rhGDNF	****
BDNF-MSC vs. rhGDNF	**	GDNF-MSC vs. GDNF/BDNF-MSC	****
GDNF-MSC vs. rhBDNF	***	GDNF-MSC vs. NGF	****
GDNF/BDNF-MSC vs. NGF	*	GDNF-MSC vs. rhBDNF	****
GDNF/BDNF-MSC vs. rhBDNF	**	GDNF/BDNF-MSC vs. NGF	*
NGF vs. rhBDNF	****	GDNF/BDNF-MSC vs. rhGDNF	****
rhBDNF vs. rhGDNF	**	NGF vs. rhBDNF	*
		NGF vs. rhGDNF	****
		rhBDNF vs. rhGDNF	****



(A)

Figure 1. (Continued)

(B)

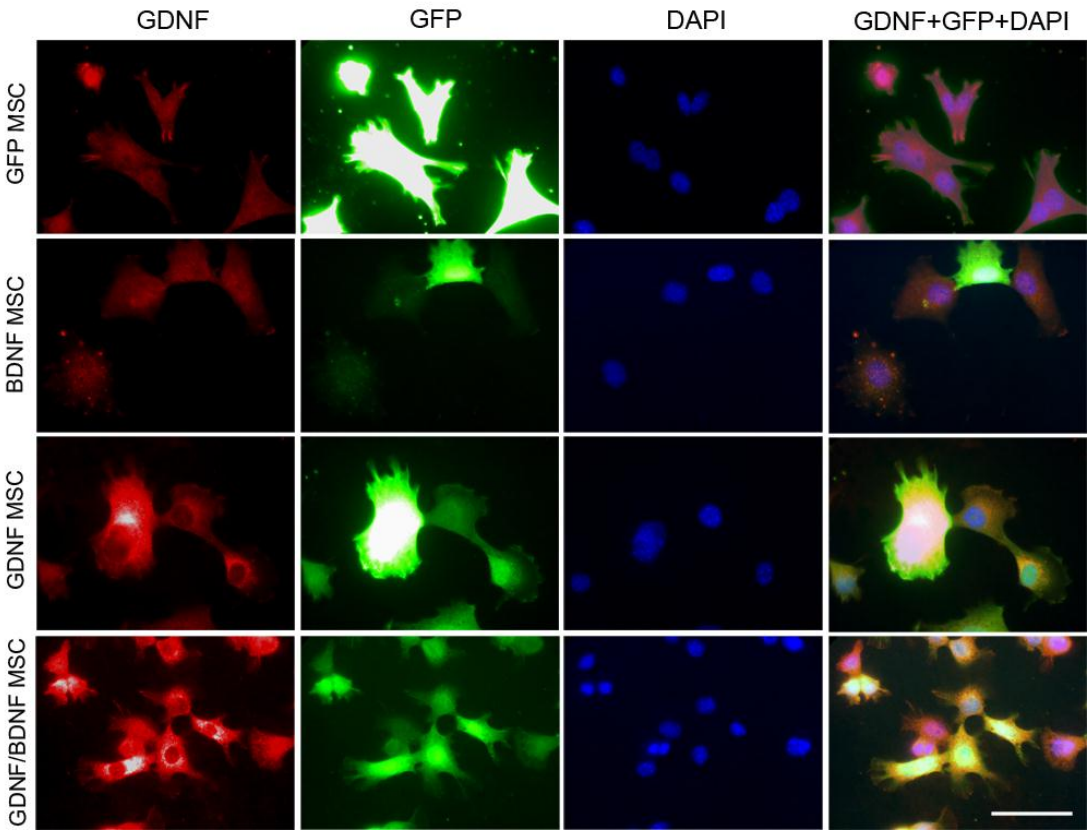


Figure 1. *In vitro* expression of BDNF and GDNF on the MSCs after lentiviral infection.

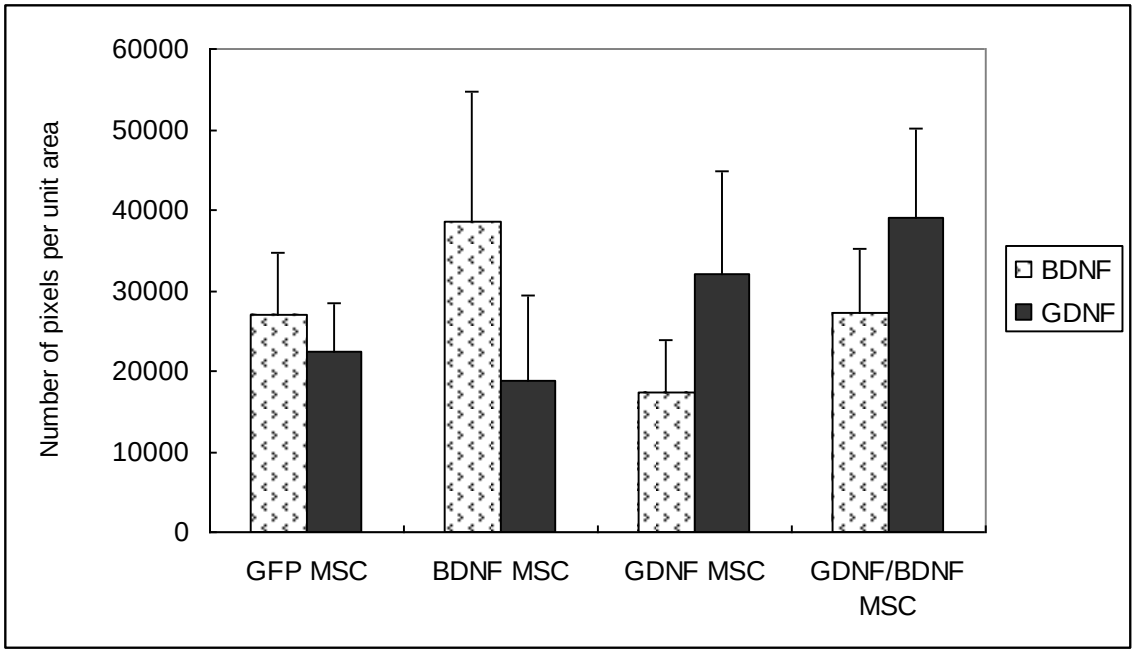


Figure 2**.** Quantification of BDNF and GDNF expression on the MSCs *in vitro.*

(A)

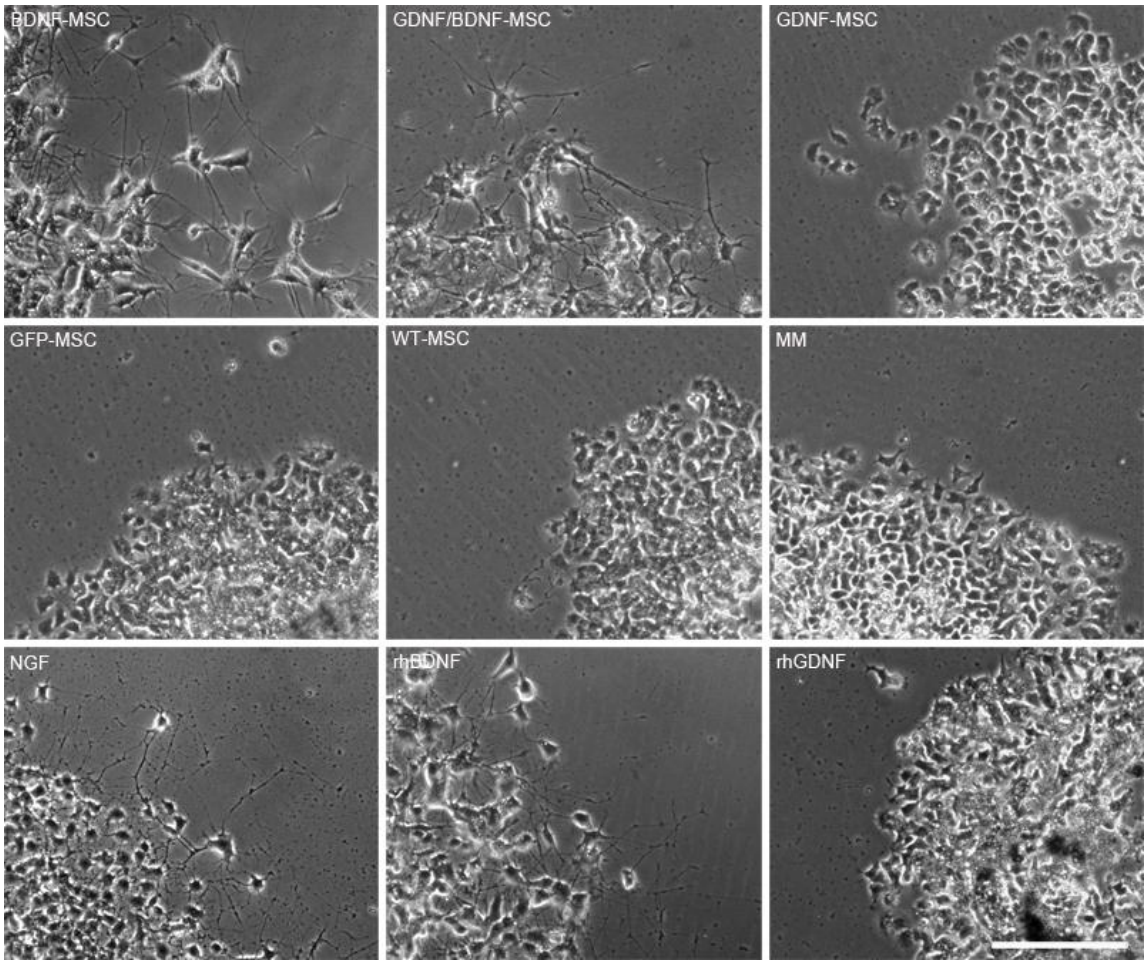


Figure 3. (Continued)

(B)

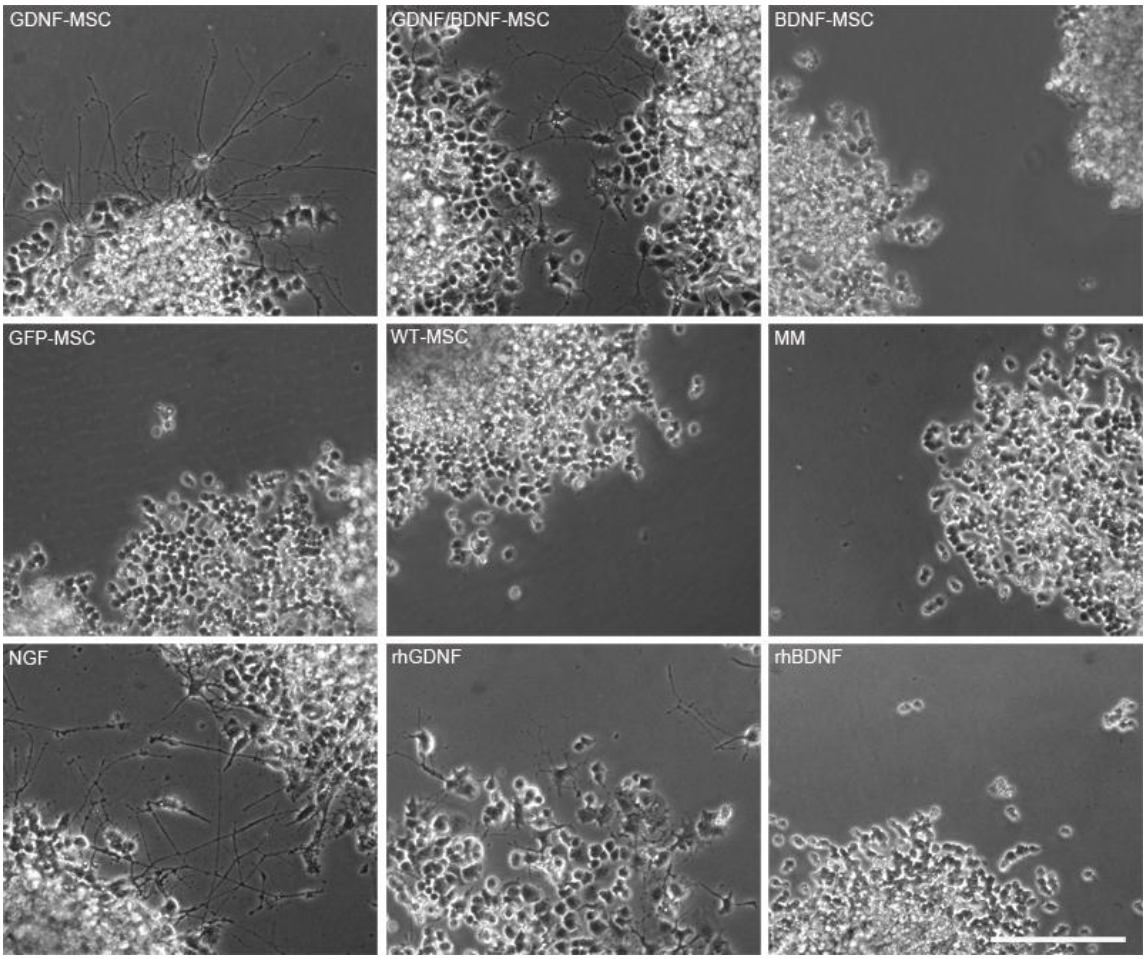
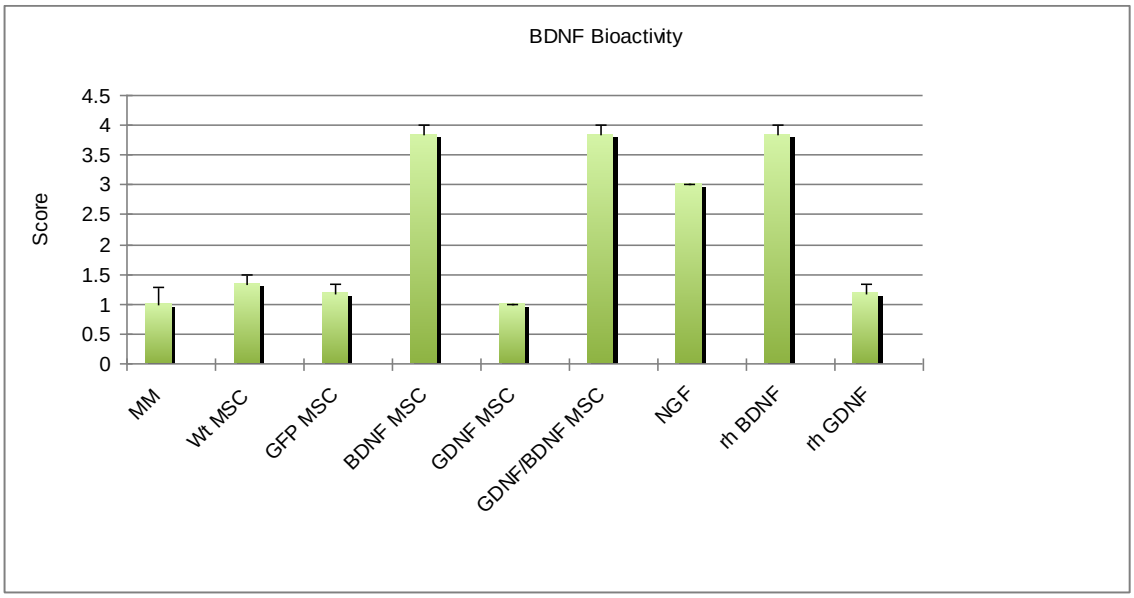


Figure 3. Representative images of bioassays.

(A)



(B)

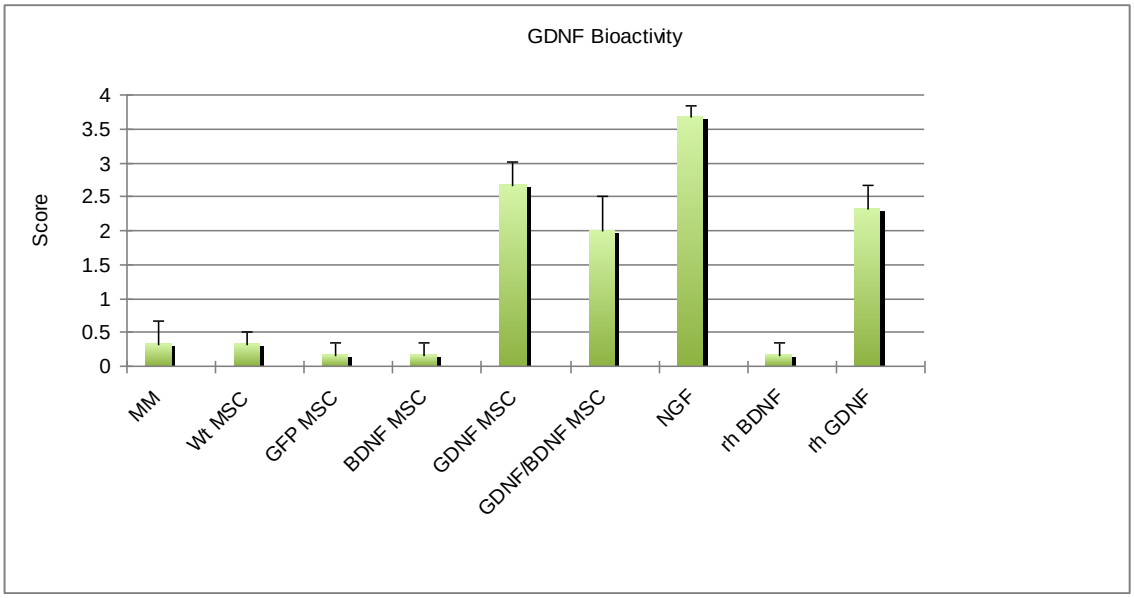


Figure 4. *In vitro* assessment of bioactivity of BDNF and GDNF.

CHAPTER 4: FUNCTIONAL AND HISTOLOGICAL EVALUATIONS OF THE RETINA AND OPTIC NERVE OF DBA/2J MICE AFTER MSC TRANSPLANTATION

This chapter is a part of the paper to be submitted to Stem Cell Research.

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Abstract

The purpose of this study was to develop neuroprotective strategies to rescue neurodegenerative retinas by stem cell-based therapy. Glaucoma, the second leading cause of blindness in the world, is a hereditary ocular disease, affecting about 60 million people throughout the world [1]. One significant factor that contributes to glaucoma is elevated intraocular pressure (IOP). Such hypertension causes the death of retinal ganglion cells (RGCs) and eventually leads to vision loss. However, there are no remedies to cure glaucoma, yet. As a potential solution to rescue degenerating retinas, stem cells have been investigated extensively and the effects of morphological neuroprotection or functional recovery have been reported [2-5]. Our previous study showed mesenchymal stem cells (MSCs) releasing brain-derived neurotrophic factor (BDNF) played a significant role to

rescue degenerating retinas, following ocular transplant in a chronic rat model of optic neuropathy [5]. In the present study we have investigated the potential effects on morphological and functional neuroprotection, using a chronic model of hereditary glaucoma, DBA/2J mice, by performing ocular injection of bone marrow-derived MSCs. Following an intraocular transplant, the engineered MSCs survived up to four months post transplant (PT) in adult host retinas without immunosuppression. Our histological examinations showed no cell proliferation in the host retina during all time points examined. In addition, we observed a potential role of MSCs on the alleviation of glial activation after MSC transplantation into the host retina. There was no noticeable decrease on the numbers of NeuN- and choline acetyltransferase (ChAT)-IR cells between 12 and 16 weeks post transplant (PT). Also, no significant difference was found on the proportions of both NeuN- and ChAT-IR cells between OS and OD in all groups. Howerver, there was a trend towards protecting NeuN-IR cells in the ganglion cell layer (GCL) by glial cell-derived neurotrophic factor (GDNF)- and GDNF/BDNF-MSCs. In addition, analysis of retinal architecture, assessed by retinal thickness, indicates potential neuroprotection on the inner retina mostly by GDNF/BDNF-MSCs.

Taken together, trends in the data suggest transplantation of MSCs, which secrete both BDNF and GDNF, is beneficial to the preservation of the host inner retina as well as retinal ganglion cells. This study provides an experimental strategy towards clinical applications in terms of using MSCs as a potential autograft, and long-term neuroprotection via neurotrophic factor delivery for retinodegenerative diseases.

Introduction

Today, millions of people in the U.S. suffer from ocular diseases, which eventually can lead to vision loss. Glaucoma, the second leading cause of blindness in the U.S., is a hereditary ocular disease, affecting about four million Americans. Elevated IOP is one of the most significant risk factors contributing to glaucoma and RGC death is a major problem causing irreversible vision loss [6, 7]. Neurotrophic factors have been investigated and used in developing strategies to rescue injured/or degenerative retinas, since It has been reported that ocular hypertension contributes to the deprivation of neurotrophic factors by blocking retrograde transport [8, 9]. BDNF stimulated regrowth of RGC axons [10] and increased the survival of RGCs [11] *in vivo*. GDNF also has been shown to have the ability to promote RGC survival in degenerating retinas [12, 13].

Engineering MSCs as cellular vehicles for neurotrophic factor delivery is a rational strategy towards overcoming challenging issues, such as sustained long-term secretion of bioactive NTs and repetitive injections into the eye. MSCs have shown neuroprotective effects on the survival of RGCs and/or their axons following intravitreal transplantation into the eyes with experimental glaucoma [3-5]. Previous studies have shown bone marrow-derived MSCs injected into the vitreous cavity exerted neuroprotective effects on the RGC axons in a rat glaucoma model [4]. In addition, MSCs engineered to express BDNF rescued degenerating retinas structurally, as well as functionally [5]. So far, no study has shown significant neuroprotective effects, both structurally and functionally, on glaucomatous models for a long-term period, greater than 2 months post transplant.

DBA/2J mice are a hereditary, spontaneous glaucoma model and a very good model organism to study experimental glaucoma, because these mice are well-investigated and has characteristics of human glaucoma, including age-related progression of IOP elevation, RGC death, and excavation of the optic nerve head [14]. DBA/2J mice have shown a linear increase of IOP from 2 to 10 months of age, and the IOP is correlated with the preservation of the optic nerve, as well as corneal thickness [15]. Furthermore, it has been shown that around 13 months of age the number of RGCs and their axons, examined by retrograde tracers, decreased significantly. However, the number of NeuN-immunoreactive (IR) RGCs was not significantly reduced until 18 months of age [16]. Studies conducted with DBA/2J mice have demonstrated that the glial lamina, which corresponds to the lamina cribrosa in humans, and proximal to the laminar region are the first areas of axon degeneration in the optic nerve [17, 18].

It has been suggested that functional deficits in RGCs precede structural abnormalities and cell death [16, 19]. Pattern electroretinogram (PERG) provides a noninvasive electrophysiological assessment of visual function. Usefulness of PERG has been demonstrated as a tool to monitor the dysfunction of RGCs in DBA/2J mice [20]. It was also reported that DBA/2J mice showed a progressive decrease of PERG amplitude, starting after 3 months of age and diminished to noise level by 11 months of age [21].

Many glaucomatous characteristics of morphological changes in the eyes of DBA/2J mice have been reported. Correlation between corneal thickness with IOP was revealed in DBA/2J mice. Increased corneal thickness, regardless of age, was observed with increased IOP. Moreover, decrease in axonal density was correlated with increased corneal thickness. The results indicated that corneal thickness is a good predictor of optic nerve degeneration

[15]. In addition, the timecourse of RGC changes in DBA/2 mice has also been examined. The deficits in axonal transport, found after 6 months age, precedes axonal loss and cell loss, observed at about 13- and 18-month old mice, respectively [16]. Furthermore, along with the degeneration of RGCs, glial changes in the retina and optic nerve are also crucial factors involved in the progression of glaucoma. Increased reactivity on the retinal astrocytes and Müller cells, and increased number of microglia were observed in aging DBA/2J mice after 3- to 4-months of age [22, 23]. In addition, in the optic nerves of aged DBA/2J mice, there was an opposite correlation between astrocyte activation and axon labeling with antibodies directed against phosphorylated neurofilament. This astrocyte reactivity was observed in the early degeneration process and was followed by a later loss of oligodendrocytes [24]. The loss of oligodendrocytes occurred coincidently with axonal loss. Also, a proliferation of oligodendrocyte precursor cells was observed [24]. Microglial activation was found in severely degenerated optic nerves, and phagocytic activity of microglia was activated after axonal degeneration [24].

Thus, we will examine morphological changes of the DBA/2J retinas following MSC transplantation, mainly focusing on microglial and Müller glial changes, as well as RGC changes. These can be important indicators to monitor glaucomatous degeneration. The aim of the studies described in this paper are to investigate the long-term effects of BDNF- and GDNF-secreting mouse MSCs in a mouse model of experimental glaucoma, seeking structural and functional protection on degenerating retinas. We have employed both functional and morphological studies to evaluate our strategy for neuroprotection. We show that MSCs can survive up to 4 months PT *in vivo*, following intraocular transplantation. The recipient eyes showed reduced glial activation and a pattern of increased thickness of retinal

layers compared with the control, non-transplanted fellow eyes. More importantly, our results suggest transplanted MSCs may play an important role in the neuroprotection of RGCs in the host eyes.

Overall, this study provides the first evidence of long-term survival of engineered MSCs in adult retina of DBA/2J mice. In addition, our results suggest bone marrow-derived MSCs can function as a potent cellular vehicle to deliver neurotrophic factors into the glaucomatous eyes and preserve the host retina.

Materials and Methods

Animals

Female DBA/2J mice, obtained from the Jackson Laboratory, at 7-8 months of age were used for MSC transplantation. The animals were kept in a constant environment (temperature: 22°C; humidity: 20 %; 14/10-hour light-dark cycle) with food and water provided ad libitum. The mice were allowed to rest for 7 days after arrival from the supplier to let them recover from transportation and adapt to the new environment before performing intraocular injection. All animal procedures for this study adhered to the provisions of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and had the approval of the Iowa State University Institutional Animal Care and Use Committee, and were performed in accordance with committee guidelines.

Mouse mesenchymal stem cells (MSCs)

Mouse MSCs isolated from the bone marrow of adult C57BL/6 mice were obtained from Tulane Center for Gene Therapy (New Orleans, LA) and maintained as an adherent cell line in Iscove's Modified Dulbecco's Medium (IMDM; 12440–046; Invitrogen, Carlsbad, CA) containing 10% hybridoma-qualified FBS (Cat #-SH30396.03, Hyclone, Logan, UT), 10% equine serum (Cat #- SH3007403, Hyclone, Logan, UT), 2 mM L-glutamine, and 10,000 U/mL penicillin, 10,000 µg/mL streptomycin and maintained at 37 ℃and CO_2 level.. When cultures reached 70–80% confluence, MSCs were gently detached from the flask using a 0.05% trypsin and 0.1% EDTA solution (Invitrogen) and were pelleted at 500 rpm for 5 minutes. MSCs were subsequently plated into 75 cm^2 flasks at low confluence. Cultures were maintained by supplementing the flask with fresh medium every other day.

Engineering MSCs *Ex Vivo* with lentiviral vectors

MSCs were infected using lentiviral vectors to engineer them to secrete brain-derived neurotrophic factor (BDNF; human cDNA) and glial cell-derived neurotrophic factor (GDNF; human cDNA), as previously reported [25]. Briefly, MSCs were plated in six-well plates at a density of 10,000 cells per well and were allowed to adhere for a day. After adhering to the plate, the growth medium in each well was replaced with IMDM, containing 2% FBS and 12 µg/mL Sequa-brene (152667; Sigma-Aldrich, St. Louis, MO). Separate lentiviral vector's encoding BDNF (LV-BDNF; CMV-BDNF-IRESGFP), GDNF (LV-GDNF; CMV-GDNF-IRESGFP), and green fluorescence protein (GFP, LV-GFP; CMV-GFP) were added separately to MSCs at a multiplicity of infection (MOI) of 1,000 for each

vector. Media containing viral particles were removed after 8 hours of incubation and fresh growth medium was added. When confluence reached 70–80%, MSCs were transferred to a flask for subsequent maintenance. Subcultures were performed as described above. MSCs, that expressed strong green fluorescence, were collected by fluorescence-activated cell sorting (FACS) using an equipment FACSAria III (BD Biosciences, San Jose, CA) controlled by BD FACSDiva software (version 6.1.3). To sort MSCs by FACS, each BDNF- and GDNF-MSCs were collected in separate 15 ml tubes. Each sample tubes contained 3 to 5 million MSCs in 0.5 ml culture media. Then, MSCs with strong green fluorescence were collected in separate catch tubes containing 1 ml FBS. Additionally, GDNF/BDNF-MSCs were generated by infecting GDNF-MSCs, which were previously FACS sorted with the LV-BDNF constructs at a MOI of 1,000.

Cell transplantation

To perform intraocular injections, MSCs were collected by gently detaching the cells with a 0.05% trypsin and 0.1% EDTA solution (Invitrogen, Carlsbad, CA), and pelleted at 500 rpm for 5 minutes. Supernatant media were removed and the pellet was resuspended with Earle's Balanced Salt Solution (EBSS, Invitrogen, Carlsbad, CA) at a concentration of 50,000 cells per µl. A beveled glass microelectrode attached to a Hamilton syringe via polyvinyl chloride tubing was used to inject 2 µl of MSC cell suspension intravitreally. Only the left eye served as an experimental group and the right eye served as a fellow, control eye. Five different groups of injections were included; (1) BDNF-GFP-MSCs, (2) GDNF-GFP-

MSCs, (3) GDNF/BDNF-MSCs, (4) GFP-MSCs as a cell control, and (5) Earle's Balanced Salt Solution (EBSS) as an injection control.

Measurement of intraocular pressure (IOP)

A TonoLab rebound tonometer (Colonial Medical Supply, Franconia, NH) was used to monitor the IOP in both experimental and control eyes, following the procedures described in the manufacturer's manual. Briefly, mice were anesthetized with 1 L/min O_2 and 3.5% isoflurane for initial induction, and maintained at 1.5% isoflurane through IOP measurement. Measurement was performed 3-4 minutes after anesthesia induction. The TonoLab acquires six rebounds of the probe from the corneal surface and calculates a mean of the middle four measurements. Body temperature was maintained on a heating pad during measurements.

Pattern electroretinogram (PERG)

To evaluate functional RGCs, PERG was used to measure the retinal potential. The mice were anesthetized with 1 L/min O_2 and 3.5% isoflurane for initial induction, and maintained at 1.5% isoflurane through PERG recording. Before PERG recording was started, the mice were dark-adapted for 10 min. After induction of anesthesia, the mice were placed on a stainless steel recording table equipped with a hot water-based warming platform to maintain body temperature. One percent tropicamide was applied to the cornea. Reference and ground electrodes were placed subcutaneously on the forehead and tail of the animal, respectively. A gold recording electrode (Roland Consult, Brandenburg, Germany) was

gently placed on the cornea and a drop of methylcellulose was used to maintain contact between the electrode and cornea. The mouse's head was positioned 15 cm from the stimulus monitor. Stimuli were evoked using alternating, reversing, black and white vertical stimuli delivered on a CRT monitor with a ERG system (Reti-Port System; Roland Consult). Stimuli of 0.02 cycles per degree (cpd), 1 Hz frequency, 200 averaged signals with cut off filter frequencies of 1-30 Hz were delivered to maximize PERG amplitude. For analysis, amplitudes from peak to trough of the waveforms were evaluated.

Tissue preparation and immunohistochemistry

The mice were deeply anesthetized with isoflurane and quickly euthanized by cervical dislocation. The eyes and optic nerves were fixed with 4% paraformaldehyde (pH 7.4). The optic nerves were dissected from the eye globes and embedded in paraffin for subsequent procedures. The eye globes were cryoprotected in 30% sucrose and embedded in the Optimum Cutting Temperature (OCT) compound (Tissue-Tek, Torrance, CA). Frozen retinal sections were prepared in 16 µm thickness and examined for immunohistochemistry analysis. A panel of primary antibodies was used for histological examinations. The tissue sections were incubated in a blocking solution containing 5% normal donkey serum and 0.2% Triton X-100 (Fisher Scientific), followed by incubation with primary antibodies overnight at 4°C. The primary antibodies used were anti-GFAP (1:500, Fisher Scientific, Pittsburgh, PA), anti-Iba1 (1:400, Wako, Richmond, VA), anti-BDNF (1:200, Santa Cruz Biotechnology, Dallas, TX), anti-GDNF (1:200, Santa Cruz Biotechnology, Dallas, TX), anti-phospho-histone H3 (pH3) (1:1,000, Abcam, Cambridge, MA), anti-caspase 3 (1:200,

Millipore, Billerica, Massachusetts), anti-GFP (1:400, Millipore, Billerica, Massachusetts), anti-NF68 (1:200, Abcam, Cambridge, MA), anti-neurofilament (NF) 200 (1:200, Sigma-Aldrich, St. Louis, MO), and GFAP-Cy3 conjugated (1:1,000, Sigma-Aldrich, St. Louis, MO). After rinsing in phosphate buffered saline (PBS), the sections were incubated with the secondary antibodies conjugated to Cy3 (1:500, Jackson ImmunoResearch, West Grove, PA), FITC (1:500, Jackson ImmunoResearch, West Grove, PA), or Cy5 (1:500, Jackson ImmunoResearch, West Grove, PA). Cell nuclei were stained with 4', 6-diamidino-2-phenylindole, dilactate (DAPI), diluted at 1:2,000 in PBS, and applied for 30 minutes. Preparations were rinsed and then mounted onto microscope slides using an anti-fade mounting medium (Gel Mount; Biomeda Corp., Foster City, CA). Negative controls were performed in parallel by omission of the primary antibodies. No antibody labeling was observed in the controls.

Quantification of retinal layer thickness

To evaluate the effect of the transplanted MSCs on the preservation of the host retinal architecture, the thickness of retinal layers was measured. Three medial cross-sections for each eye were analyzed. Retinal sections were stained with a cocktail of Phalloidin-conjugated with Alexa Fluor 488 (1:1,500, Invitrogen, Carlsbad, CA) and DAPI (1:2,000). The thickness measurements for each retinal layer were compiled for both peripheral and a central retinal image taken of each cross-section. Using ImageJ software, standardized grid coordinates were generated within each image. Then, three lines, aligning perpendicular to the retinal layers and distributed equidistant from each other, were analyzed within each image. The thickness of the outer and inner nuclear layers was measured based on DAPI

staining. Phalloidin staining was used to measure the thickness of the outer and inner plexiform layers, and the ganglion cell layer together with the nerve fiber layer. Analysis was performed in a masked manner.

Retinal imaging and statistics

The retinal images were photographed, using an upright fluorescence microscope (Nikon Microphot FXA) equipped with a Retiga 2000R digital camera controlled by QCapture software (QImaging, Surrey, British Columbia, Canada). Figure plates were prepared using Photoshop CS2. For quantitative analyses, data were reported as means ± S.E.M. Statistical analysis was performed by using Student's *t*-test, paired *t*-test, and one-way ANOVA (GraphPad, San Diego, CA). $P < 0.05$ was considered significant.

Results

IOP measurement after MSC transplantation

Elevated IOP is a significant risk factor for glaucoma. The DBA/2J mice developed glaucomatous characteristics, as IOP increased starting around 6 months of age [26]. Thus, we monitored IOP changes in both experimental and the fellow eyes after MSC transplantation as time progressed (Table 1). IOP was recorded at 2, 4, and 7 weeks post transplant on the DBA/2J mice. At 2 weeks post transplant (PT), the IOP of OS that received EBSS injection (16.11 ± 1.933 mmHg) was significantly higher than that for OD (12.11 ± 1.124 mmHg) (P value < 0.05, paired t-test). However, all the other groups that received MSC transplants did not show significant differences of IOP between treated and untreated

eyes (P value **>** 0.05, paired t-test). Also, IOP changes at 4 and 7 weeks PT were not significant between OS and OD (P value **>** 0.05, paired t-test).

Overall, we found a decreasing pattern of IOP in the eyes of the injection groups compared to that of untreated eyes during the 7 weeks following injection. Also, there was a transient increase of IOP in OS, following EBSS injection, at 2 weeks PT.

Assessment of functional RGCs

The RGC function of DBA/2J mice was evaluated, using pattern electroretinogram (PERG) after MSC transplantation (Table 2). The functional recordings were performed at 2, 4, and 7 weeks PT along with IOP measurements. The amplitudes (P50-N95) ranged from 3 to 8 μV, in general, and no significant differences were found between OS and OD. Only one group, eyes with EBSS injection at 4 weeks PT, showed significantly reduced amplitude compared with the fellow eyes; OS (4.158 ± 0.850 μV) and OD (7.435 ± 1.013 μV) (P value <0.05, paired t-test). In addition to the amplitude, latency (P50) was also analyzed. No significant differences were found between the eyes at the time points examined (data not shown).

Overall, PERG amplitude during the early time points after injection did not show statistically different changes for any of the conditions, except EBSS injected group at 2 weeks PT. OS with EBSS injection at the time point showed a transient decrease of amplitude, compared to that of fellow eyes.

Survival of transplanted MSCs in adult DBA/2J mice

We examined the capacity of MSCs to survive *in vivo*, following intraocular transplantation into the adult retina of DBA/2J mice. Based on green fluorescent protein (GFP) expression, we found MSCs survived in the host retina up to 16 weeks PT. Representative images are shown in Figure 1. Many GFP-MSCs at 3 weeks PT (Figure 1; upper panel) and GDNF/BDNF-MSCs at 2 weeks PT (Figure 1; bottom panel) were located along the posterior segment of the retina. No MSC integration into the host retina was observed at the time-points examined.

We observed considerable variability in MSC survival between individuals in the same MSC transplant group. Also, the survival ability appeared to vary among the different MSC populations (Table 3). GFP- and BDNF-MSCs were detected after four months PT, while GDNF/BDNF-MSCs were detected up to three months PT. Based on GFP-expression, we detected no GDNF-MSCs surviving in the host retinas at any of the time points examined. Mice, receiving EBSS injection (N = 6), were not included in Table 3.

Qualitative analysis of retinal structure of the host retinas after MSC transplantation

The initiation and progression of glaucoma can be monitored by histological examinations of the retina. Thus, we performed a qualitative analysis to examine phenotypic changes on host retinas after MSC transplantation. We found a large proportion of the eyes receiving GDNF-MSCs showed reduced glial activation, represented by GFAP- and Iba1-IR,

compared with the fellow eyes (Figure 2). Such a reduction of activated glia was not prominent in those eyes in the other MSC transplant groups. Along with changes in glial cell activation, we also examined possible retinal cell proliferation. Using an antibody directed against phospho-histone H3 (pH3-IR), we detected little to no cell proliferation (pH3-IR) in the host retinas at all time points examined.

Assessment of retinal layer thickness

We evaluated whether MSC transplantation would preserve retinal architecture as DBA/2J mice aged. In this analysis we measured the thickness of both inner and outer retina as well as the whole retinal layers for both OS and OD at 16 weeks PT (Fig. 3). Our results showed that MSC-injected OS showed a pattern of increased thickness of the inner retina (NFL + GCL + IPL) on both central and peripheral retinas than for the fellow eyes (Fig. 3A). However, the pattern of increased thickness in OS was not observed on the outer retina (OPL+ONL) (Fig. 3B). For the inner retina, the central region in GDNF/BDNF-MSC injected OS retained significantly thicker layer than the fellow eye (OS: 17.62 ± 3.94 μm vs. OD: 9.69 ± 1.39 μm; N = 2 - 3, P = 0.024, paired t-test).

When analyzing the thickness of whole retinal layers, there was no statistical difference between OS and OD for all the injection groups (Fig. 3C; P value $>$ 0.05, paired t-test). We also analyzed changes of the ratio of inner and outer retinal portion to the whole retinal layers. The proportional thickness of the inner and outer retinas was not significantly different between OS and OD in any groups examined (Fig. 3D; P value $>$ 0.05, paired t-test). Also, peripheral retinas did not show significant changes in their proportions (Fig. 3E).

Our results suggest that GDNF/BDNF-MSCs contributed to the preservation of the host inner retina in aging DBA/2J mice.

Changes of retinal ganglion cells and choline acetyltransferase (ChAT)-IR amacrine cells in the host retina

We assessed whether transplanted MSCs played a neuroprotective role on the RGC survival as well as a subpopulation of amacrine cells in the host retina of aging DBA/2J mice. We examined the number of NeuN-IR cells in the ganglion cell layer (GCL) and ChAT-IR cells in the GCL and INL to see whether RGC survival and ChAT-IR amacrine cells, respectively, was influenced by MSC transplantation. Firstly, there was no noticeable decrease on the numbers of NeuN- or ChAT-IR cells between 12 and 16 weeks post transplant (PT) (data not shown). Also, no significant difference was found on the proportions of both NeuN- and ChAT-IR cells between OS and OD in all groups (Fig. 4). However, there was a trend towards neuroprotection of NeuN-IR cells in the GCL by GDNF- and GDNF/BDNF-MSCs at both 12 and 16 weeks PT.

Discussion

In the studies presented here, we have demonstrated that engineered MSCs are capable of surviving in the adult retina of DBA/2J mice for a relatively long period of time. MSCs survive up to 4 months after intraocular transplantation into the host retina without immune suppression. To our knowledge, this is the first study implementing MSC transplantation and investigating possible benefits of MSC survival in the DBA/2J retina. We modified MSCs genetically by performing lentiviral infection for genes of interest. MSCs

were engineered to produce and secrete BDNF and/or GDNF together with GFP using lentiviral vectors. MSCs were easily detectable, due to an endogenous expression of GFP. Four different types of MSCs were generated and used for our transplant study- GFP-, BDNF-, GDNF-, and GDNF/BDNF-MSCs. We observed differences in cell survival between the different MSC types. Unexpectedly, no GDNF-MSCs were detected in the host retina at any of the time points examined in this study. We can assume that GDNF, along with allografts, activated the immune system in the host retina and resulted in the poor survival rate for GDNF-MSCs [27]. In contrast, GFP- and BDNF-MSCs survived until 16 weeks PT in the host retinas. GDNF/BDNF-MSCs were detected until 12 weeks PT, but not at 16 weeks PT.

To evaluate physiological and functional changes of glaucomatous eyes, IOP and PERG amplitude were monitored. Left eye (OS- oculus sinister) received the transplants, and right eye (OD- oculus dexter) served as an internal, fellow control eye. During the early time points following MSC transplantation (7 weeks post transplant), we found some changes of IOP and PERG amplitude which were statistically significant between OS and OD. However, such changes appeared to be a transient phenomenon, possibly resulting from the injection procedures.

The PERG recordings were conducted up to seven weeks following MSC transplants. Consistent PERG recordings were not possible beyond that time point due to corneal calcification and extensive corneal vascularization as DBA/2J mice age [28]. Large variations of PERG amplitudes at 7 weeks were attributed to such corneal problems and this also resulted in decreased sample sizes. Based on the physiological and functional assessments, there was neither noticeable neuroprotection nor deleterious effects following

an intraocular injection during the early time points by 7 weeks PT.

Following physiological and functional assessments, the influence of MSC transplant on the retina was examined by histological methods. We found no or little dividing cells, revealed by pH3-IR, exist in the host retina at all time points examined. This result suggests no vitreous hemorrhage and retinal detachment occurred following our injection [29-31].

We also showed transplanted MSCs played a potential role in alleviating glial (Iba1- and GFAP-IR) activation. It has been suggested that microglial activation is a potential contributor to the onset or progression of glaucoma in DBA/2J mice [23]. Other studies have also revealed a relationship of microglia with axonal integrity and transport. Activated microglia obstruct axonal transport of synaptic vesicle precursors and microglial activation can induce neurite beading, which precedes neuronal death [32-34]. In addition, an elevated expression of GFAP in Müller cells was found in the glaucomatous retina, indicating the contribution of Müller cells to the pathological processes [35, 36]. Thus, our results suggest that the reduction of glial activation resulted from neuroprotective roles of transplanted MSCs *in vivo*.

The influence of transplanted MSCs on the axons and astrocytes in the glial lamina of optic nerves has also been investigated (not presented here). Our results may reveal that neurotrophic factor-secreting MSCs contribute to the rescue of light and/or heavy neurofilaments, and to the maintaining of axonal transport and integrity. It has been reported that the heavy neurofilament (NFH) isoform, but not the light neurofilament (NFL) isoform, are decreased in the lamina cribrosa and postlaminar regions in pig eyes with elevated IOP [37]. A study in DBA/2J mice demonstrated that immunoreactivity for both NFH and NFL

was notably reduced in the optic nerve in the aging mice [16]. In addition, previous studies have reported reactive astrocytes in glaucomatous eyes. Reactive astrocytes were observed in glaucomatous optic nerve head, showing thick and enlarged processes [38]. Also, there was about a three-times increase in the number of astrocytes in the optic nerves of old DBA/2J mice [24].

The survival of RGCs in the retina is another crucial factor, since the number of RGCs decreased significantly at around 13 months of age, when examined by retrograde tracers [16]. In addition to RGCs, subpopulation of amacrines (Cholinergic (ChAT) and GABAergic (GABA) amacrine cells) also showed significantly decreased numbers in DBA/2J mice compared to that of B6 mice [39]. Surprisingly, about 60% of cells in the GCL are displaced amacrine cells in the mouse retina [40]. In the retina of DBA/2J mice, 3.6% of NeuN-IR cells were ChAT-IR displaced amacrines at 3 months of age and 16.2% were ChAT-IR amacrine cells at 12 and 18 month of age [16].

Although we did not observe significant differences on the proportions of both NeuN- and ChAT-IR cells between OS and OD in all groups, there was a trend towards protection of NeuN-IR cells in the GCL by GDNF- and GDNF/BDNF-MSCs at both 12 and 16 weeks PT.

We also studied whether engineered MSCs could preserve the retinal architecture of the host retina. Previous studies have shown other retinal layers, in addition to GCL, are also impacted due to pathological processes in glaucoma. DBA/2J mice had a thinner retinal thickness on the OPL and IPL, as well as reduced numbers of cells in the INL and GCL, compared with those of B6 mice [39]. Also, synaptic ribbons of the rods disintegrated in aging DBA2J mice [41].

We discovered the recipient eyes with GDNF/BDNF-MSCs maintained significantly thicker proportion of the central inner retina, compared with the fellow eyes. These findings suggest that the combination of GDNF and BDNF may have resulted in a neuroprotective effect leading to a preservation of the inner part of the host central retina, but not the outer retina, following MSC transplantation in the aging DBA/2J mice. The significance found on only central retina and not the peripheral retina was possibly due to the distribution of surviving MSCs *in vivo*.

When evaluating absolute values of retinal thickness, we found a pattern of increased thickness of the OS inner retina (NFL + GCL + IPL) on both central and peripheral regions, compared with the fellow eyes, although the differences were not statistically different. For the thickness of whole retinal layers, OS from GDNF-MSC group showed significantly lower values (P value = 0.0464) on the central region, compared to the fellow eyes. It is possible that the OS (transplanted eyes) were undergoing a more rapid degeneration compared with the fellow, control eyes, and that any GDNF released from MSCs could not be delivered to the outer retina efficiently to prevent them from undergoing rapid degeneration.

Possible reasons for neuroprotective effects on the inner retina, but not on the outer retina, may be partly attributed to the localization of neurotrophic factor receptors within the inner retina. The expression of TrkB, a BDNF receptor, is expressed in majority of RGCs, amacrines, and at the vitreal border of the INL in the vertebrate retina [42]. For GDNF receptors, Ret is expressed in horizontal, amacrine, and RGCs and not in photoreceptors or Müller cells. GFRα1 expression is found in horizontal, amacrine, and RGCs. GFRα2 is localized in amacrine and RGCs,in the mouse retina [43]. As a future study, analyzing the retinal thickness of young D2 and the same age of B6 mice, as control groups, would be

useful for comparison. Also, we found a trend that GDNF-secreting MSCs showed a potential neuroprotection on the host retina, although we could not detect the cells in the host eyes for a sustained period of time following transplantation. That possibly suggests that a burst release of GDNF during the early period of time following transplantation resulted in neuroprotection on the host retina.

Taken together, our results suggest the transplanted MSCs are potential cellular vehicles to survive for a long-term period in the glaucomatous retinas. Also, trends in the data suggest transplantation of MSCs, mostly secreting both BDNF and GDNF, is beneficial to the preservation of the host inner retina as well as retinal ganglion cells.

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Figure Legends

Table 1. Measurement of IOP changes recorded at 2, 4, and 7 weeks post transplant on DBA/2J mice following MSC transplantation. Significant differences of IOP values between control and experimental eyes are indicated with an asterisk (*); (1) OS vs. OD in EBSS group at 2 weeks post injection, (2) non-treated control vs. injection groups at 2 weeks post transplant (PT), and (3) non-treated control vs. injection groups at 7 weeks PT. Pattern of decreasing IOP in the injection group compared with the fellow eyes, as time progressed.

(Numbers: Average ± S.E.M.; *P value <0.05, paired t-test; Abbreviation: OS-oculus sinister, left eye; OD-oculus dexter, right eye)

Table 2. Recordings of PERG amplitudes (P50-N95) performed at 2, 4, and 7 weeks PT in DBA/2J mice following MSC transplantation. Significant differences of amplitude between control and experimental eyes are indicated with an asterisk (*); between the OS and OD in EBSS group at 4 weeks PT. In addition to amplitude, latency (P50) also analyzed and no significant differences were found between the eyes (data not shown). (Numbers: Average ± S.E.M.; *P value <0.05, paired t-test)

Table 3. Survival of transplanted MSCs in adult DBA/2J host retina.

Numbers of mice with transplanted MSCs at different time points of tissue collection are summarized. Transplanted MSCs survived up to 16 weeks PT in the adult DBA/2J retinas. Different survival ratio was observed between individuals in the same MSC group as well as between different MSC populations. No GDNF MSCs survived in host retinas at any time point examined. Mice receiving EBSS injection (N = 6) not included in this table. Numbers in bold type mean number of mice with surviving MSCs out of the total number of mice in the group.

Figure 1. Survival of transplanted MSCs in adult DBA/2J host retina. Many GFP-MSCs at 3 weeks PT (A) and GDNF/BDNF-MSCs at 2 weeks PT (B) found along the posterior segment

of host retina. MSCs retained a strong expression of GFP and often localized along the inside of the host retina. Abbreviations: ONH-optic nerve head; DAPI: 4', 6-diamidino-2-phenylindole, dilactate; DIC: differential interference contrast. Scale bars are 50 microns.

Figure 2. Potential role of MSCs on the reduction of glial activation after MSC transplantation into host retina. Some eyes, that received MSCs, showed relatively decreased level of glial activation, compared with the fellow eyes. (A and B) Retinas stained with GFAP antibody. Eyes received GDNF-MSCs and collected at 4 weeks PT shown. (C and D) Retinas stained with Iba1 antibody. Eyes received GDNF-MSCs and collected at 12 weeks PT shown as examples.

A and C: OS, B and D: OD. Scale bars are 50 microns. Abbreviations: ONL- outer nuclear layer; INL- inner nuclear layer; IPL- inner plexiform layer; GCL- ganglion cell layer.

Figure 3. Assessment of retinal layer thickness. The thickness of both inner and outer retina as well as the whole retinal layers were quantified for both OS and OD at 16 weeks PT. (A) The thickness of the inner retina (NFL + GCL + IPL): OS, which received an MSC injection, showed a pattern of increased thickness of the inner retina on both central and peripheral retinas, compared with the fellow eyes. The central region in GDNF/BDNF-MSC injected OS showed a significant difference on the thickness between OS and OD (OS: 17.62 ± 3.94 μm vs. OD: 9.69 ± 1.39 μm; P = 0.024, paired t-test). (B) The thickness of the outer retina (OPL+ONL): The increased pattern of the thickness in OS was not observed on the outer

retina. (C) The thickness of whole retinal layers: None of the injection groups showed statistical differences of the thickness between OS and OD.

(D and E) The ratio of inner and outer retinal portion to the whole retinal layers:. The proportional thickness of the inner and outer retinas was not significantly different between OS and OD in any groups examined. Also, peripheral retinas did not show significant changes of the proportions.

Figure 4. RGC survival in the DBA/2J host retina following MSC transplantation.

Percentages of NeuN- and ChAT-IR cells in the host GCL were assessed at 12 weeks- (A) and 16 weeks-(B) PT. No significant difference was found on the proportions of both NeuN- and ChAT-IR cells between OS and OD in all groups. However, there was a trend towards protecting NeuN-IR cells in the GCL by GDNF- and GDNF/BDNF-MSCs at both 12 and 16 weeks PT.

Tables and Figures

Table 1. Measurement of IOP changes recorded at 2, 4, and 7 weeks post transplant on the DBA/2J mice following MSC transplantation.

Weeks post transplant	**2**		**4**		**7**	
Transplant group	OS	OD	OS	OD	OS	OD
EBSS	16.11 * (± 1.933)	12.11 * (± 1.124)	12.13 (± 1.922)	11.88 (± 1.846)	14.33 (± 2.418)	17.67 (± 1.563)
GFP MSC	19.27 (± 2.141)	15.36 (± 1.416)	15.6 (± 2.495)	18.2 (± 2.004)	10.44 (± 0.93)	12.56 (± 1.435)
BDNF MSC	18.36 (± 2.674)	13.27 (± 0.915)	12 (± 1.653)	14.8 (± 1.533)	12.5 (± 2.252)	13.33 (± 1.716)
GDNF MSC	17.2 (± 1.123)	16.5 (±1.833)	14 (± 2.435)	16.38 (± 1.752)	12.5 (± 1.565)	17 (± 3.13)
GDNF/BDNF MSC	14.27 (± 1.192)	14.18 (± 1.577)	17.88 (± 3.528)	13.57 (± 1.771)	13.5 (± 1.384)	15.6 (± 3.027)

Table 2. Recordings of PERG amplitudes (P50-N95) performed at 2, 4, and 7 weeks post transplant in DBA/2J mice following MSC transplantation.

Weeks post transplant	**2**		**4**		**7**	
Transplant group	OS	OD	OS	OD	OS	OD
EBSS	5.52 (± 0.910)	4.189 (± 0.819)	4.158 * (± 0.850)	7.435 * (± 1.013)	16.87 (± 2.137)	11 (± 5.878)
GFP MSC	5.132 (± 0.964)	3.656 (± 0.733)	5.585 (± 0.988)	6.267 (± 1.634)	7.247 (± 3.587)	5.508 (± 1.346)
BDNF MSC	5.539 (± 0.823)	4.622 (± 1.378)	8.783 (± 2.067)	7.808 (± 1.303)	13.5 (± 3.674)	8.862 (± 2.862)
GDNF MSC	4.185 (± 1.036)	4.357 (± 0.632)	5.362 (± 1.177)	4.832 (± 1.058)	4.19 (± 0.93)	5.695 (± 0.085)
GDNF/BDNF MSC	3.735 (± 0.609)	4.475 (± 0.910)	2.94 (± 1.61)	4.483 (± 1.57)	13.71 (± 6.047)	2.72

Table 3. Survival of transplanted MSCs in the adult DBA/2J host retina.

Transplant Groups	Number of Mice	Weeks Post Transplant		
		< 12	12	16
GFP MSC	9	**1/2**	**1/3**	**3/4**
BDNF MSC	9	**1/1**	**1/3**	**2/5**
GDNF MSC	9	**0/3**	**0/3**	**0/3**
GDNF/BDNF MSC	9	**2/4**	**2/3**	**0/2**

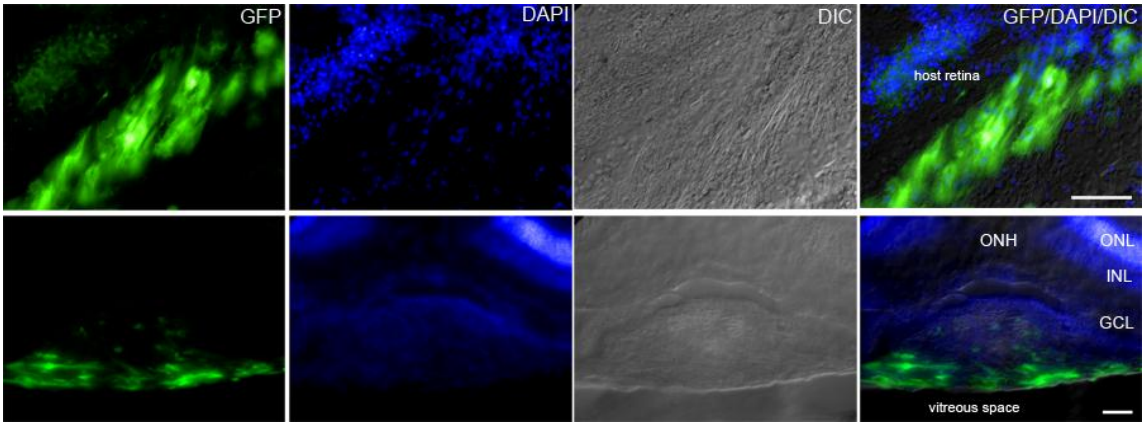


Figure 1. Survival of transplanted MSCs in the adult DBA/2J host retina.

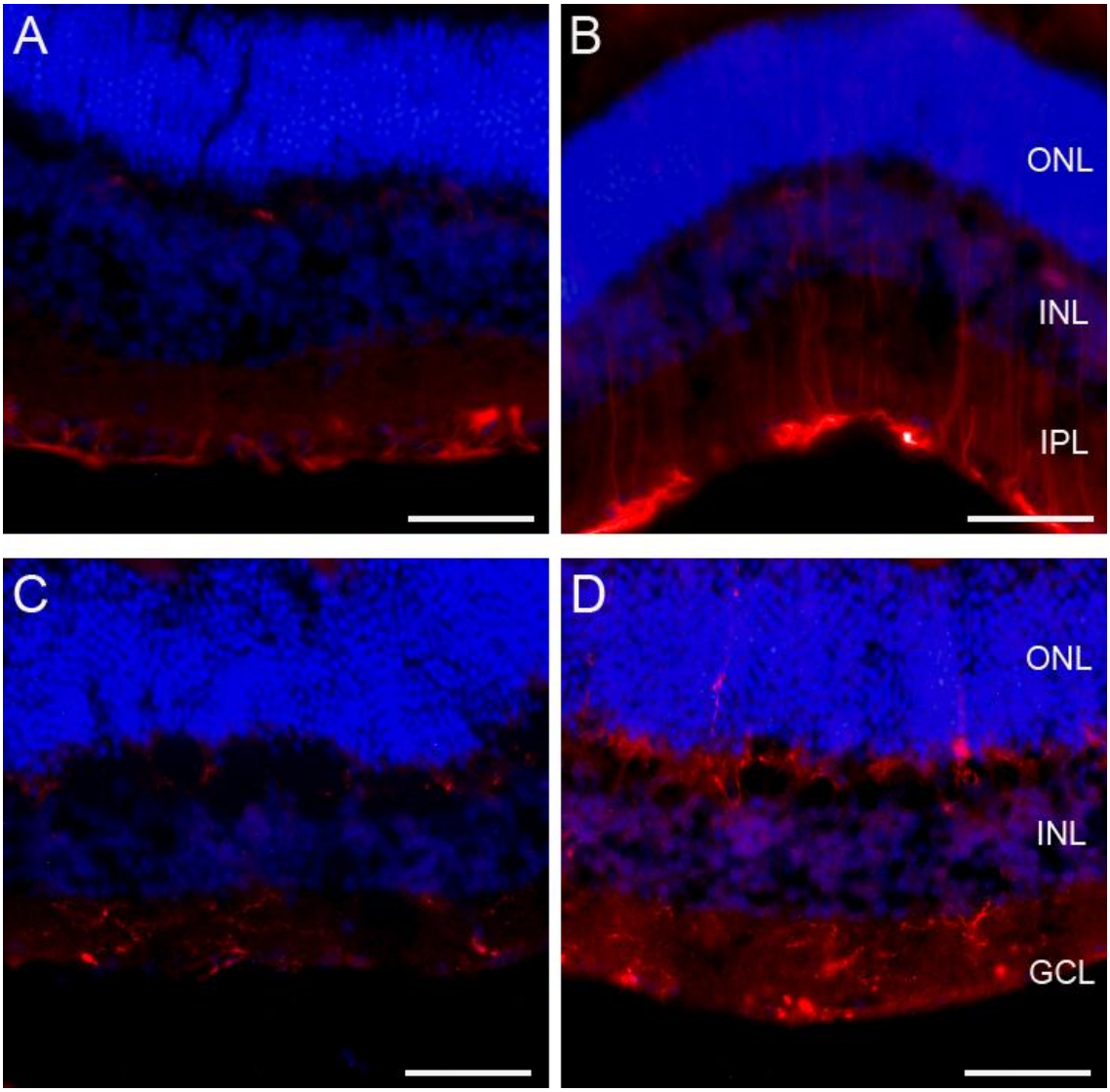
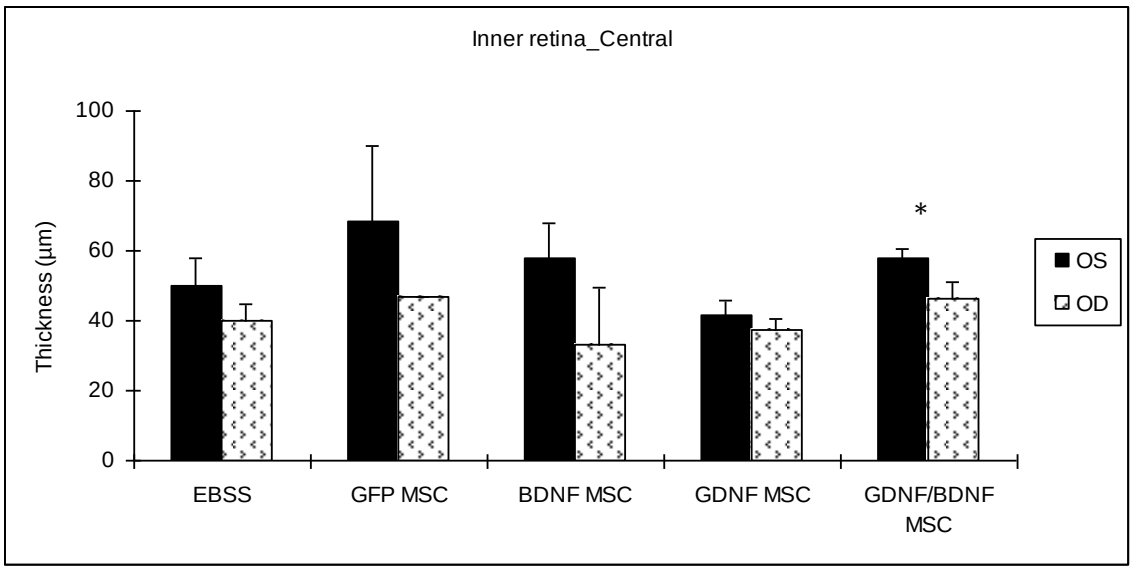


Figure 2. Potential role of MSCs on the reduction of glial activation after MSC transplantation into the host retina.

(A) Inner retina (GCL + NFL + IPL)



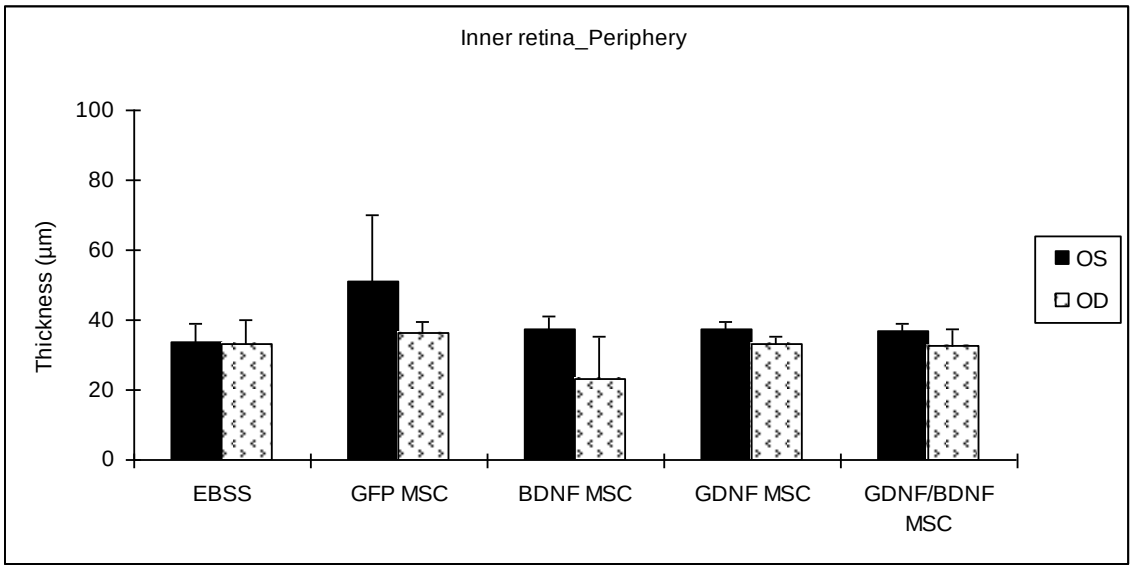
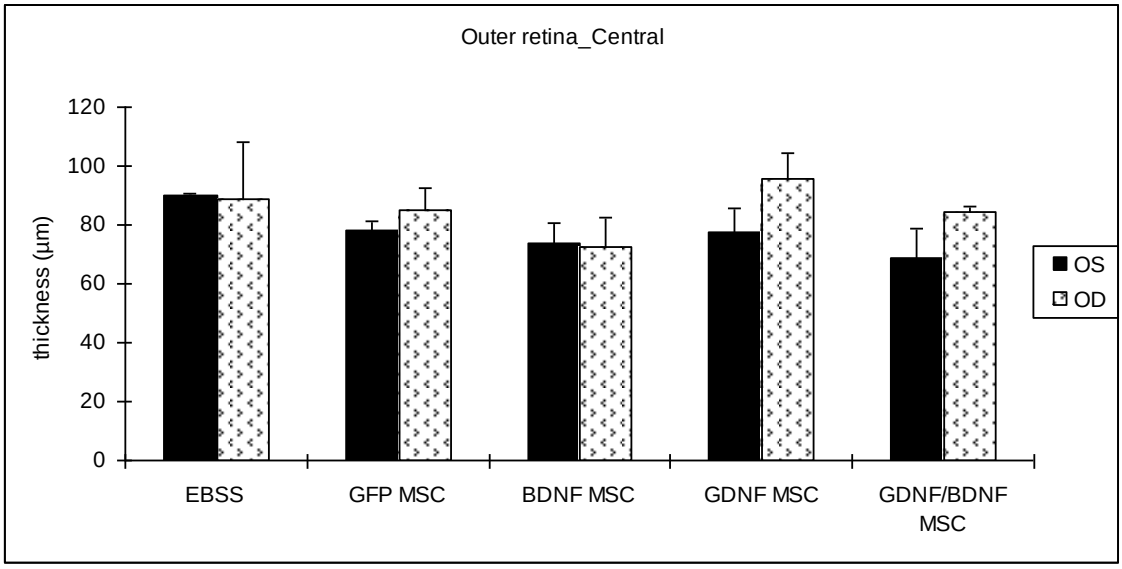


Figure 3. (Continued)

(B) Outer retina (OPL+ONL)



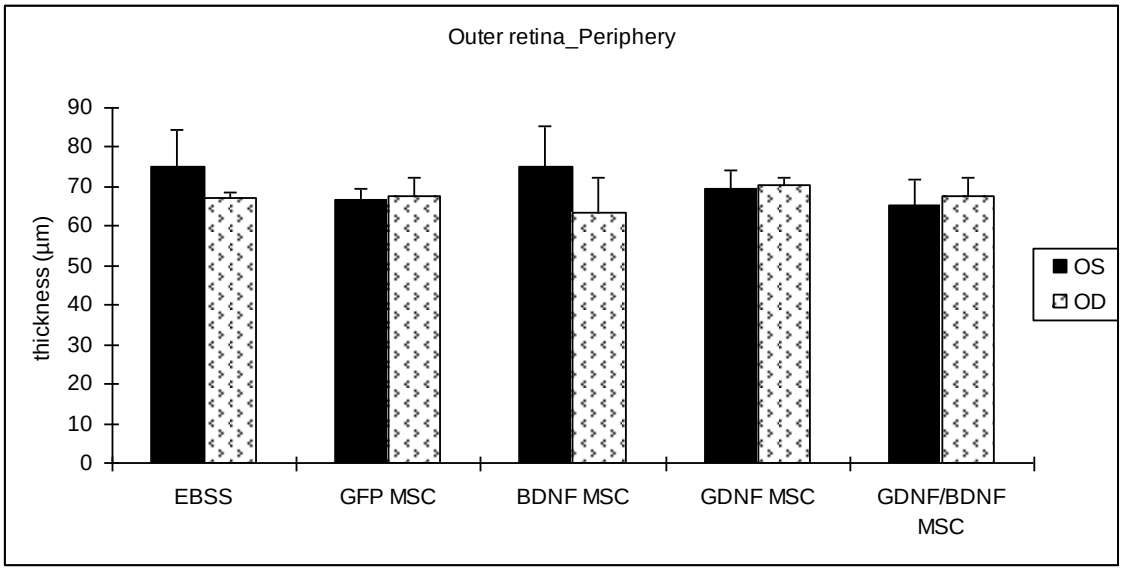
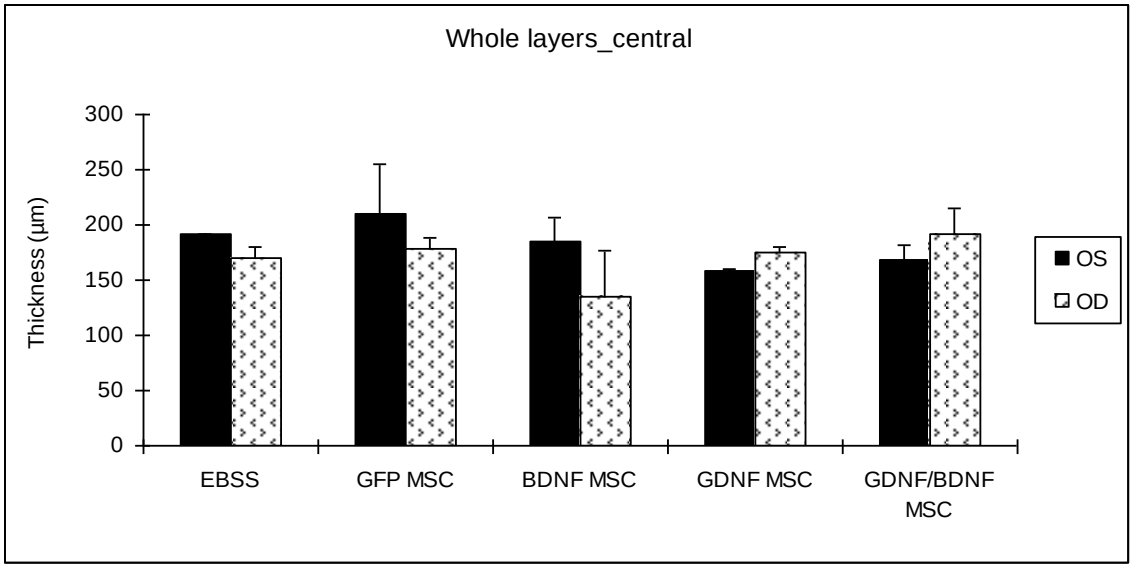


Figure 3. (Continued)

(C) Whole retinal layers



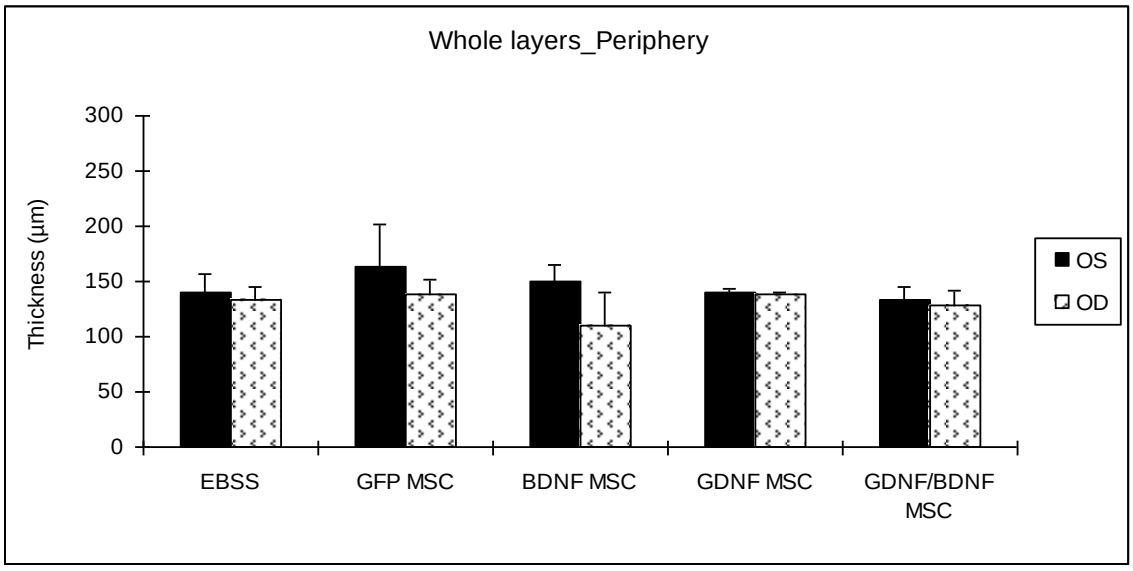
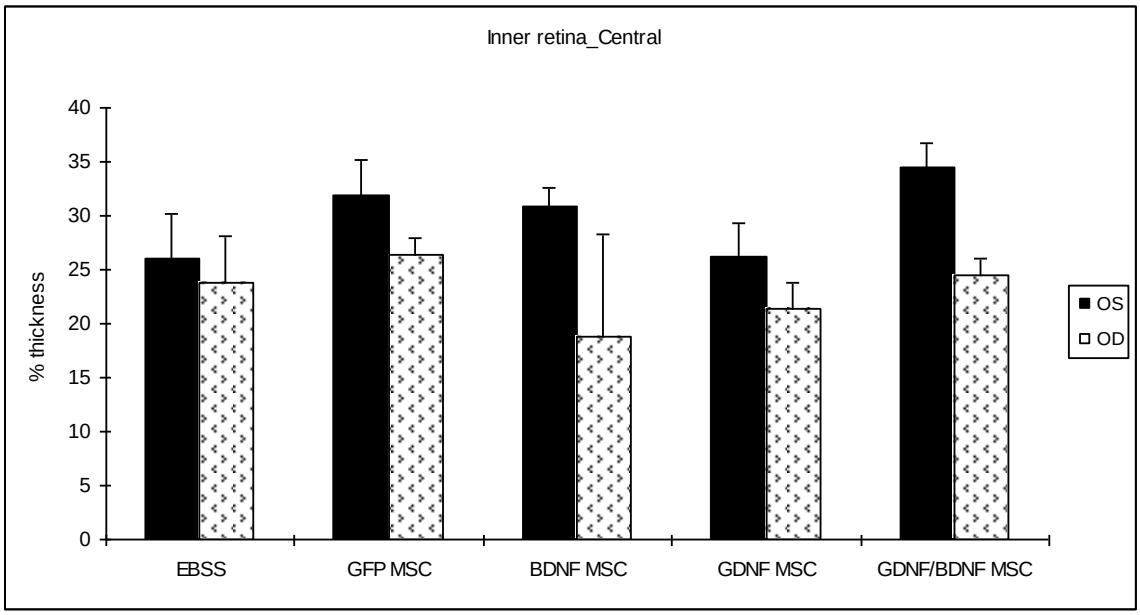


Figure 3. (Continued)

(D) Inner retina (GCL + NFL + IPL)



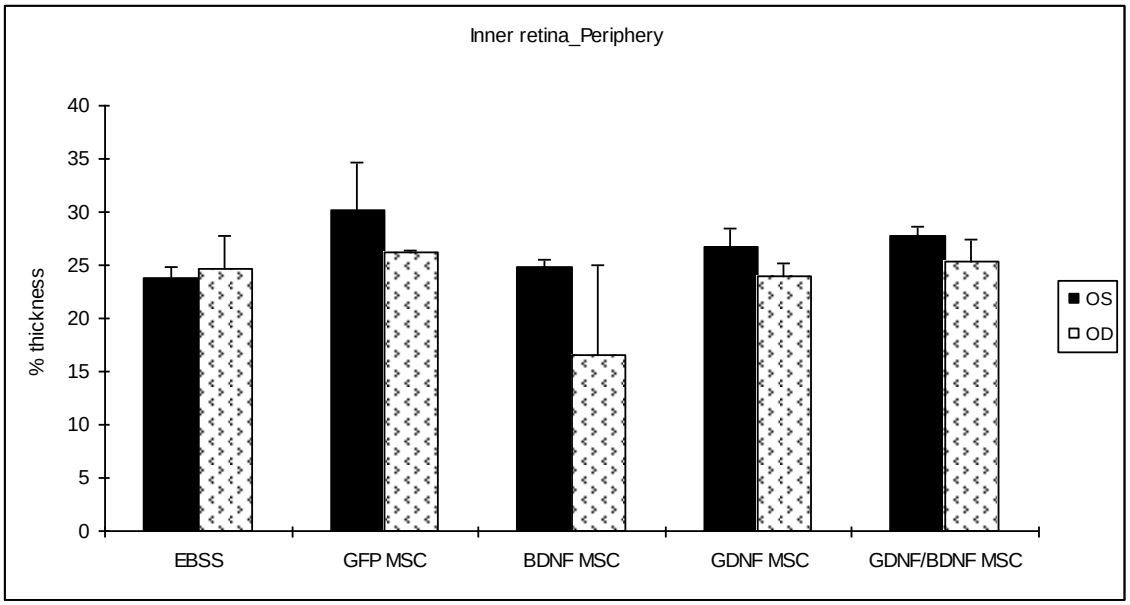
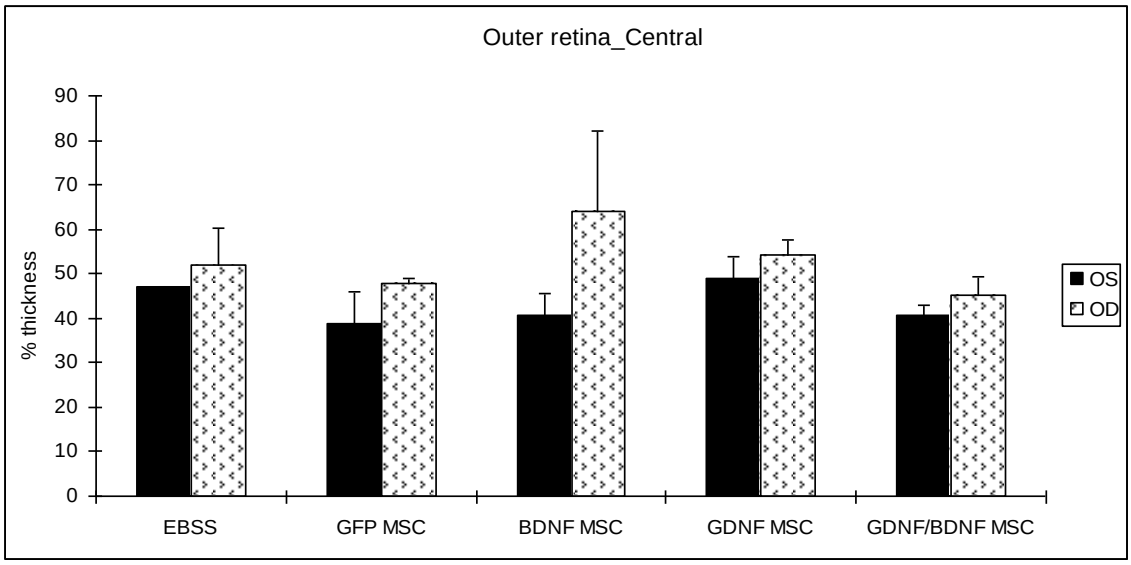


Figure 3. (Continued)

(E) Outer retina (OPL + ONL)



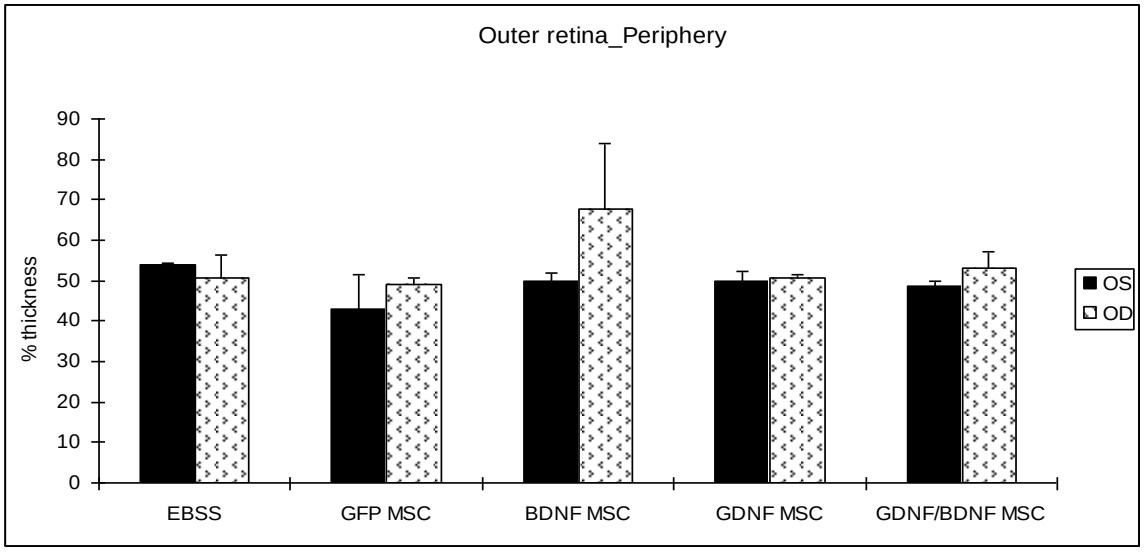
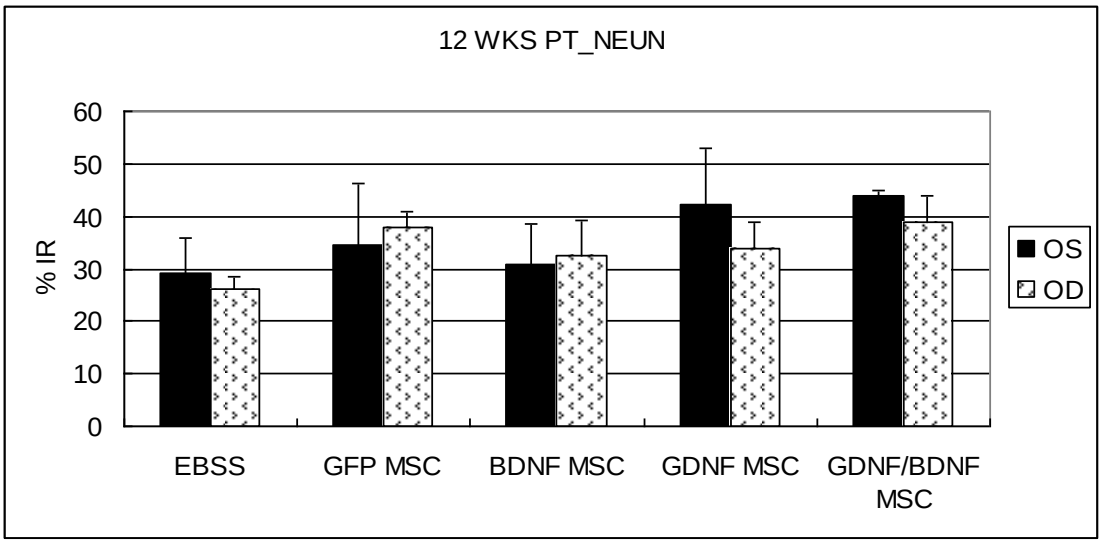


Figure 3. Assessment of retinal layer thickness

(A) 12 weeks PT



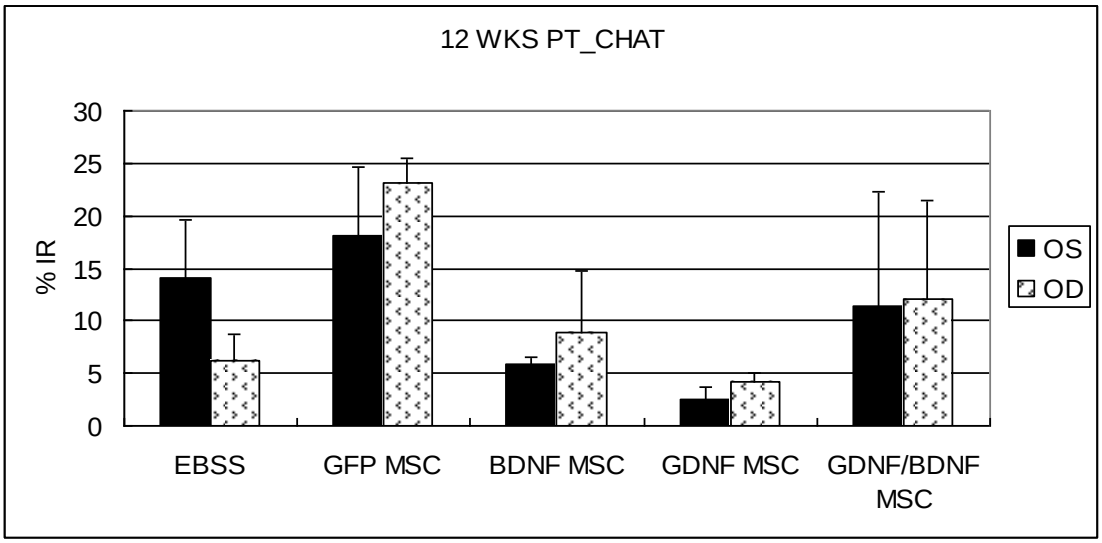


Figure 4. (Continued)

(B) 16 weeks PT

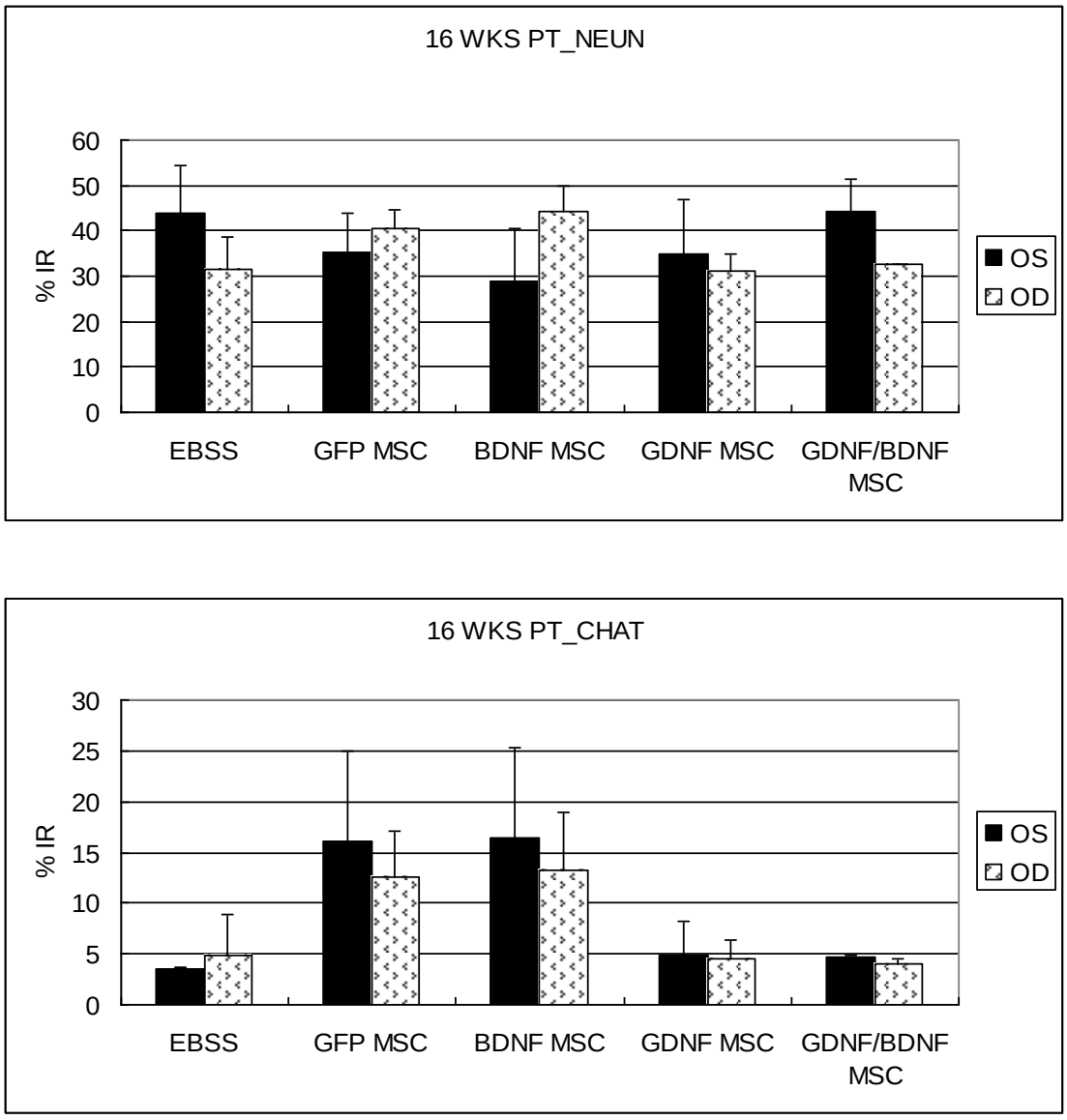


Figure 4. RGC survival in the DBA/2J host retina following MSC transplantation.

CHAPTER 5: HISTOLOGICAL EVALUATIONS OF RETINAS FROM MYOCILIN (MYOC)-MUTANT MICE AFTER MSC TRANSPLANTATION

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Abstract

The purpose of this preliminary study was to investigate the potential neuroprotective effects of BDNF-secreting MSCs on the retina of MYOC-mice. Mutations in MYOC are found in 3-4% of people with primary open-angle glaucoma (POAG). A transgenic mouse model, Tg-MYOC^{Y437H}, was generated by using a human MYOC gene modified to introduce a point mutation. The mice are an additional model of chronic glaucoma. *In vitro* studies indicate that mutant Myocilin accumulates in the endoplasmic reticulum (ER), and results in ER stress and cytotoxicity on the cells of the trabecular meshwork. *In vivo* mechanisms that lead to IOP elevation in this mutant mouse are not understood yet, but such ER stress may be involved in the development of IOP elevation [1-3]. It has been known that BDNF functions as a survival factor for neurons *in vitro* as well as *in vivo* [4-9]. In addition, BDNF signaling is involved in the suppression of ER stress and neuronal apoptosis [10-12].

In this study, we employed two types of genetically modified MSCs, BDNF/GFP-MSCs and GFP-MSCs, to deliver BDNF into the host retina via cellular vehicle. Each MSC populations were intravitreally injected into 8 week-old eyes of MYOC mice. Then, histological examinations were performed at 11.5 months post transplant (PT). Both BDNF- and GFP-MSCs survived up to 11.5 months PT in the adult host retina. However, transplanted MSCs did not significantly affect the number of surviving RGCs (NeuN-immunoreactive), comparing the experimental and control eyes. Our immunohistochemical staining revealed myocilin is primarily expressed on the trabecular meshwork (TM), sclera, and ciliary body. When comparing the levels of myocilin protein on the TM, the recipient eyes displayed stronger immunoreactivity (IR) against myocilin than the fellow eyes.

Most interestingly, to our knowledge, these results provide the first evidence that MSCs can survive approximately a year in the adult retina of MYOC-mice following intraocular transplantation. This suggests MSCs are a potential cellular vehicle to deliver trophic factors to the host retina for a long-term period. In addition to this preliminary study, further examination is required to increase our knowledge on developing therapeutic strategies for glaucoma.

Introduction

Of the three major types of glaucoma, primary open angle glaucoma (POAG) is the most common [13]. In humans, there is an inheritance factor in primary open-angle glaucoma (POAG) and mutations in MYOC are found in 3-4% of people with POAG [14]. Myocilin is a secreted glycoprotein highly expressed in the trabecular meshwork, ciliary body, sclera,

and iris, as well as expressed in the retina and the optic nerve head at low amounts [15]. It has been suggested that myocilin plays a role in the interaction of cells with the matrix, but the functions of myocilin are not fully understood, yet [14-17].

As an additional transgenic model of glaucoma, myocilin (MYOC)-mutant mice have been developed and characterized [3, 18]. A transgenic mouse mode, Tg-MYOC^{Y437H}, was generated by using the human MYOC gene, modified to introduce a point mutation [3]. It has been reported that mutant myocilin accumulates in the endoplasmic reticulum (ER), and results in ER stress and cytotoxicity on the cells of the TM. Such ER stress leads to the development of IOP elevation [1-3]. Tg-MYOC^{Y437H} mice were characterized and showed glaucomatous phenotypes, such as IOP elevation, axon degeneration, and death of RGCs [3].

Neurotrophic factors have been investigated and utilized in developing strategies of rescuing injured/or degenerative retinas, since it is known the retrograde transport of BDNF was interrupted in eyes of experimental glaucoma [19, 20]. In addition, the absence of endogenous BDNF and NT-4/5 was shown in the superior retina and optic nerve head of hypertensive rat eyes during the time of RGC death [29]. It has been demonstrated that BDNF stimulated regrowth of RGC axons [7] and increased the survival of RGCs [8] *in vivo*. Moreover, BDNF delivered via MSCs to glaucomatous eyes with chronic ocular hypertension provided both functional and structural neuroprotection on the host retina and optic nerve [9].

Bone marrow-derived MSCs are a potential source for stem cell-based therapy. Several advantages include 1) easy isolation, 2) significant plasticity, 3) no ethical concerns,

4) a potential autologous transplant, and 5) availability of engineering to produce a cellular vehicle for delivering NFs for a long-term period.

Engineering MSCs to make cellular vehicles for neurotrophic factor delivery allows us to overcome challenging issues, such as sustained long-term secretion of bioactive NTs and repetitive injections into the eye. MSCs have shown morphological neuroprotective effects on the survival of RGCs/or their axons following intravitreal transplantation into the eyes of experimental glaucoma [21, 22]. In addition to morphological protection, functional rescue of the RGCs is required and is a more crucial issue to demonstrate the capacity of MSCs as a neuroprotectant. It appears functional deficits in RGCs precede structural abnormalities and cell death [23, 24].

Therefore, the purpose of the study in this chapter is to investigate potential neuroprotective effects of engineered MSCs on the eyes of MYOC-mice. We performed histological examinations on the host retina, following MSC transplantation to assess our neuroprotective strategy. For the first time, we demonstrated engineered MSCs can survive up to a year in the adult retina of MYOC-mice without immune suppression. We quantified the number of surviving RGCs of the host retina to determine the potential neuroprotective effect of MSCs. There was no significant change of NeuN-immunoreactive (IR) cells on the ganglion cell layer (GCL).

Materials and Methods

Animals

In collaboration with the University of Iowa, 11 MYOC-mice (8-weeks old) were used for this transplant experiment. This transgenic mouse model (Tg-MYOCY437H) expresses a human MYOC gene containing the Y437H mutation, as previously described [3]. Mice were housed and bred at the Research Animal Facility of the University of Iowa. All animal studies were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by Iowa State University Institutional Animal Care and Use Committee and the University of Iowa Animal Care and Use Committee.

Mouse mesenchymal stem cells (MSCs)

Mouse MSCs isolated from the bone marrow of adult C57BL/6 mice were obtained from Tulane Center for Gene Therapy (New Orleans, LA) and maintained as an adherent cell line in Iscove's Modified Dulbecco's Medium (IMDM; 12440–046; Invitrogen, Carlsbad, CA) containing 10% hybridoma-qualified FBS (Cat #-SH30396.03, Hyclone, Logan, UT), 10% equine serum (Cat #- SH3007403, Hyclone, Logan, UT), 2 mM L-glutamine, and 10,000 U/mL penicillin, 10,000 µg/mL streptomycin. When cultures reached 70–80% confluence, MSCs were gently detached from the flask using a 0.05% trypsin and 0.1% EDTA solution (Invitrogen) and were pelleted at 500 rpm for 5 minutes. MSCs were subsequently plated into 75 flasks at low confluence. Cultures were maintained by supplementing the flask with fresh medium every other day.

Engineering MSCs *Ex Vivo* with lentiviral vectors

MSCs were infected using lentiviral vectors to engineer them to secrete brain-derived neurotrophic factor (BDNF; human cDNA) as previously reported [6]. Briefly, MSCs were plated in six-well plates at a density of 10,000 cells per well and were allowed to adhere for a day. After adhering to the plate, the growth medium in each well was replaced with IMDM, containing 2% FBS and 12 μg/mL Sequa-brene (152667; Sigma-Aldrich, St. Louis, MO). Each lentiviral vector's encoding BDNF (LV-BDNF; CMV-BDNF-IRES-GFP) and green fluorescence protein (GFP, LV-GFP; CMV-GFP) were added separately to MSCs at a multiplicity of infection (MOI) of 1,000 for each vector. Media containing viral particles were removed after 8 hours of incubation and fresh growth medium was added. When confluence reached 70–80%, MSCs were transferred to a flask for subsequent maintenance. Subcultures were performed as described above. MSCs, that expressed strong green fluorescence, were collected by fluorescence-activated cell sorting (FACS). To sort MSCs by FACS, BDNF-MSCs were collected in a 15 ml tube. The sample tube contained 3 to 5 million MSCs in 0.5 ml culture media. Then, MSCs with strong green fluorescence were collected in a catch tube containing 1 ml FBS.

Cell transplantation

To perform an intraocular injection, MSCs were collected by gently detaching with a 0.05% trypsin and 0.1% EDTA solution, and pelleted at 500 rpm for 5 minutes. The supernatant media was removed and the pellet was resuspended with Earle's Balanced Salt Solution (EBSS) at a concentration of 50,000 cells per μl. A beveled glass microelectrode attached to a Hamilton syringe was utilized to inject 2 μl of MSC cell suspension

intravitreally. The left eye served as an experimental group and the right eye was the control group. Two different groups of injections were included: (1) BDNF-GFP-MSCs (N = 6) and (2) GFP-MSCs (N = 5) as a cellular control.

Tissue preparation and immunohistochemistry

Ocular tissues were collected at 11.5 months post transplant (PT). The mice were deeply anesthetized with ketamine/xylazine and quickly euthanized by cervical dislocation. The eye globes were fixed with 4% paraformaldehyde (pH 7.4) and cryoprotected in 30% sucrose and embedded in an OCT compound (Tissue-Tek, Torrance, CA). Frozen sections were prepared in 16 µm thickness and examined for immunohistochemistry analysis. A panel of primary antibodies was used for histological examinations. Tissue sections were incubated in a blocking solution containing 5% normal donkey serum and 0.2% Triton X-100 (Fisher Scientific), followed by incubation with primary antibodies overnight at 4°C. Primary antibodies used were anti-GFAP (1:500, Fisher Scientific, Pittsburgh, PA), anti-Iba1 (1:400, Wako, Richmond, VA), anti-GFP (1:400, Millipore, Billerica, Massachusetts), and anti-phospho-histone H3 (pH3) (1:1,000, Abcam, Cambridge, MA), After rinsing in PBS, tissue sections were incubated in the secondary antibodies conjugated to Cy3 (1:500, Jackson ImmunoResearch, West Grove, PA), FITC (1:500, company), or Cy5 (1:500, company). Cell nuclei were stained with 4', 6-diamidino-2-phenylindole, dilactate (DAPI), diluted at 1:2,000 in PBS, and applied for 30 minutes. Preparations were rinsed and then covered with coverglass using an antifade mounting medium (Gel Mount; Biomeda Corp., Foster City,

CA). Negative controls were performed in parallel by omission of the primary antibodies. No antibody labeling was observed in the controls.

RGC counting

To quantify the number of surviving RGCs in the retina, we assessed the number of NeuN-IR cells in the GCL. NeuN is a nuclear protein that identifies mature neurons. It is reported that most NeuN-IR cells on the GCL are RGCs, with a small proportion of choline acetyltransferase (ChAT)-IR displaced amamcrine cells [24]. Both OS and OD from 11 mice were analyzed. NeuN-IR cells were manually counted, using ImageJ software. Three medial cross-sections from each eye were analyzed and three separate areas in each retinal section were assessed: center, periphery1, and periphery2. Imaging on the peripheral regions was performed from the very end of peripheries on each side. Analysis was performed in a masked manner.

Imaging and statistics

Retinal images were photographed using an upright fluorescence microscope (Nikon Microphot FXA) equipped with a Retiga 2000R digital camera controlled by QCapture software (QImaging, Surrey, British Columbia, Canada). Figure plates were prepared using Photoshop CS2. Data were reported as means ± S.E.M. Statistical analyses were performed by using Student's *t*-test, paired *t*-test, and one-way ANOVA (GraphPad, San Diego, CA). $P < 0.05$ was considered significant.

Results

Long-term survival of MSCs in adult eyes of MYOC-mice

We studied myocilin-mutant (MYOC) mice as an additional model of experimental glaucoma. This model develops glaucomatous phenotypes which mimick human patients', and the mice are the first transgenic model of *MYOC*-associated POAG [3]. In addition, this model does not develop iris stromal atrophy, as DBA/2J mice do. Thus, pupillometry, which evaluate pupillary light reflex, can be used as another fuctional method plus to electroretinography. Two different types of MSCs (BDNF/GFP- and GFP-MSCs) were engineered and utilized for this study, and an intraocular injection was performed on the left eye only. Then, both eyes were collected at 11.5 months PT for histological examinations. We discovered both BDNF- and GFP-MSCs survived up to 11.5 months PT in the adult host retina (Figure 1 and Table 1). MSCs were found in the posterior segment of the eye and no integration of transplanted cells into the host retinas was observed. There was variability of MSC survival between mice. Some eyes retained small or large numbers of MSCs, while others lacked the visible green MSCs. A wilt-type mouse, C57BL/6J, was included in the study and a few MSCs survived in its eye as well.

In an eye (ID 5) that contained large numbers of MSCs, we found many pH3-IR cells in the MSC population (image not shown). Immunoreactivity to pH3, which is a marker for Histone H3 phosphorylation, indicates that the cells are mitotic. However, all other OS did not show pH3-IR MSCs. Also, we have not observed any pH3-IR cells in the host retinas of all the mice examined.

Assessment of RGC survival after MSC transplantation

To evaluate the survival of retinal ganglion cells in the adult MYOC-mice following MSC transplantation, we quantified the number of NeuN-IR cells on the GCL of the retinas (Figure 2). NeuN is a nuclear protein that identifies mature neurons. It is reported that most NeuN-IR cells on the GCL are RGCs, with a small proportion of ChAT-IR displaced amamcrine cells [24]. The percentage of neuronal cells on the GCL was calculated for the NeuN-IR cells as compared to all cell nuclei stained with DAPI. Figure 3 and Table 2 shows the average percentages of surviving RGCs (NeuN-IR) of OS and OD after the experiment period of 11.5 months. Both central and peripheral retinas are analyzed from BDNF- and GFP-MSC transplant groups. No significant difference was found between experimental and control eyes, although there was a trend that showed a greater number of surviving NeuN-IR cells in the control eyes than in the treated eyes. Approximately 30 to 50% of the cells are NeuN-IR on the GCL of MYOC-mice retinas. No significant difference was shown between the percentages of neuronal cells in the GFP-MSC condition when compared to the BDNF-MSC condition.

Expression of myocilin on the ocular tissues

Since this transgenic model of glaucoma was generated by the mutation of MYOC gene, which, in turn, results in the accumulation of mutant myocilin protein on the trabecular meshwork (TM), we investigated whether MSC transplantation would influence the expression of myocilin protein on the TM. We performed immunohistochemistry using a polyclonal rabbit antibody to mouse Myoc [25] and the immunoreactivity against myocilin

was shown primarily on the TM, sclera, and ciliary body. In many cases, myocilin expression was upregulated on the TM region in the OS, compared to the fellow eyes (Figure 4).

Discussion

In this preliminary study we have demonstrated that engineered MSCs are capable of surviving in the adult retina of MYOC-mice for approximately one year following intravitreal transplant. To our knowledge, this is the first study implementing MSC transplantation and investigating possible benefits of MSC survival in the MYOC-mice retina. We provided the first evidence that demonstrates the longest time for MSC survival, following intraocular transplantation into the eyes of MYOC-mice. MSCs, visualized by green fluorescence, were found in the eyes of both transgenic and wild-type mice at 11.5 months-PT. Although we observed a larger number of cases for GFP-MSC survival than BDNF-MSC's, it is highly possible that strong GFP expression on the GFP-MSCs allowed us to detect them easier than BDNF-MSCs, which showed little or no detectable GFP.

To date, no studies have demonstrated that transplanted MSCs survive as long in the adult host eye. In a similar study on the rats with experimental glaucoma, MSCs transplanted intravitreally only survived for five weeks [21]. When comparing these results with other studies, our findings of MSC survival up to 1 year brings about an interest in finding the reason for the long life of MSCs.

We observed no dividing cells (pH3-IR) in the host retinas, which may suggest no vitreous hemorrhage and retinal detachment occurred following our injection. Only one OS (ID 5), which contained a number of GFP-MSCs, showed many pH3-IR cells in the MSC

population. That indicates some of the transplanted MSCs were still capable of proliferating in the host retina at 11.5 months post transplant. This could be possible, as the eye is an immune privileged site and provides tolerant environments for cell transplant [28].

The survival of RGCs in the retina is a crucial factor and it was reported that Tyr437His mutant mice showed 20% loss of RGCs in the peripheral retina at 18 months of age, compared to that of wild-type littermates. However, no significant RGC loss was found in the central retina [18]. From the analysis of NeuN-IR cells on the host GCL, we determined no significant difference between the percentages of surviving RGCs at both central and peripheral retinas in either transplant group as compared to the control group. A previous study [9] demonstrated BDNF-secreting MSCs transplanted into glaucomatous eyes of rats resulted in both functional and morphological preservation of the host retina, as compared to the eyes with GFP-MSC transplant. We could expect the rescue effects of BDNF-secreting MSCs on the RGCs as well as TM cells. TrkB, a BDNF receptor, is expressed in majority of RGCs [30] and TM cells [31]. Additionally, exogenous BDNF suppresses ER stress, which is a possible mechanism of TM cell death in MYOC-mice, and results in neuronal protection [32, 33]. For our results, we cannot exclude the possibility that lack of detectable neuroprotection could be caused by a small sample size and/or lack of examinations at different time-points before 11.5 months PT.

We discovered an increased level of glial activation, examined by GFAP- and Iba1-IR, in the retinas that received MSC transplant compared to the fellow eyes (data not shown). This may indicate injection of MSCs does cause an effect on the host retina, possibly because of the invasive nature of introducing a foreign substance into the eye. It has been known that the microglia function either compromises graft viability or promotes graft survival by

secreting trophic factors [26]. This still requires further investigation on the roles of microglia in retinal transplants, so we can improve our strategy for increasing graft viability.

Other studies have reported that mutant myocilin is accumulated in the TM of MYOC-mice and such accumulations were reduced by a chemical treatment—phenylbutyric acid [3, 27]. In our study, we found TM of the recipient eyes showed stronger IR against myocilin, compared to the fellow eyes. This possibly indicates transplanted MSCs affected the eye to result in increased secretion or an accumulation of myocilin on the TM, which was an unexpected result. Further studies would be required to understand *in vivo* mechanisms that resulted in this phenomenon.

In conclusion, our results provide the first evidence that MSCs could survive a year in the adult retina of MYOC-mice, following intraocular transplantation. This suggests MSCs are a potential cellular vehicle to deliver trophic factors to the host retina for a long-term period. In addition to this preliminary study, further examinations are required to increase our knowledge on developing therapeutic strategies for glaucoma.

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Figure Legends

Table 1. A summary table showing the survival of transplanted MSCs in the retina of adult MYOC-mice. (N: none, +: a few, ++: some, +++: many, ++++: a lot)

Table 2. Survival of retinal ganglion cells (NeuN-IR) in the adult MYOC-mice retina. Numerical values of the RGC survival are shown.

Figure 1. Survival of transplanted MSCs in the host retina of MYOC-mouse. A retinal section with a number of GFP-MSCs is shown. The eye from mouse ID 5 received GFP-

MSCs and retinal tissues were collected at 11.5 months PT. Scale bar = 100 μm.

Abbreviation: RPE- retinal pigment epithelium.

Figure 2. Representative images showing NeuN-IR cells in the GCL of the host retina. Both peripheral regions as well as central retina were analyzed. Red: NeuN-IR cells on the GCL; Blue: nuclei stained with DAPI. Scale bar = 100 μm.

Abbreviations: ONL- outer nuclear layer; INL- inner nuclear layer; IPL- inner plexiform layer; GCL- ganglion cell layer.

Figure 3. Survival of retinal ganglion cells (NeuN-IR) in the adult MYOC-mice retina.

The change of RGC number is quantified by counting NeuN-IR cells on the ganglion cell layer of the retinas. Graphs show the average percentage of surviving NeuN-IR cells after the experiment period of 11.5 months for both OS and OD. Central and both peripheral retinas are analyzed and no significant difference was found between the control and treatment eyes. Approximately 30 to 50 % of the cells are NeuN-IR on the GCL of MYOC-mice retinas. The *p* values for the MSC injection group as compared to controls are > 0.05. Error bars are standard error.

Figure 4. Upregulated expression of myocilin protein on the trabecular meshwork (TM) after MSC transplantation. The expression of myocilin was compared on the TM between the experimental and fellow eyes. Increased immunoreactivity of myocilin (red) are found on the TM (indicated by arrow heads) of OS, compared to that of OD.

A – C: OD, D – F: OS; Abbreviations: R- retina, CB- ciliary body, TM- trabecular meshwork; Scale bars are 100 microns.

Tables and Figures

Table 1.

Transplant	Mouse ID	Mouse Strain	Number of surviving MSCs
GFP MSC	3	C57BL/6J	+
	1	MYOC	+
	4	MYOC	N
	5	MYOC	++++
	6	MYOC	++
BDNF MSC	2	MYOC	N
	7	MYOC	N
	8	MYOC	+
	9	MYOC	+
	10	MYOC	N
	11	MYOC	N

Table 2. Survival of NeuN-IR cells in the adult MYOC-mice retina.

(A) Transplant of BDNF-MSCs

NeuN-IR	OS			OD		
	Periphery 1	Periphery 2	Central	Periphery 1	Periphery 2	Central
Number of mice	4	4	4	5	5	5
Mean	**31.96**	**31.16**	**39.82**	**39.71**	**45.93**	**42.83**
Standard Error	3.1	3.335	6.032	6.281	6.205	8.65

(B) Transplant of GFP-MSCs

NeuN-IR	OS			OD		
	Periphery 1	Periphery 2	Central	Periphery 1	Periphery 2	Central
Number of mice	1	1	1	4	4	4
Mean	**36.62**	**35.23**	**42.18**	**55.13**	**48.04**	**51.74**
Standard Error	NA	NA	NA	5.131	4.171	2.588

(NA: not applicable)

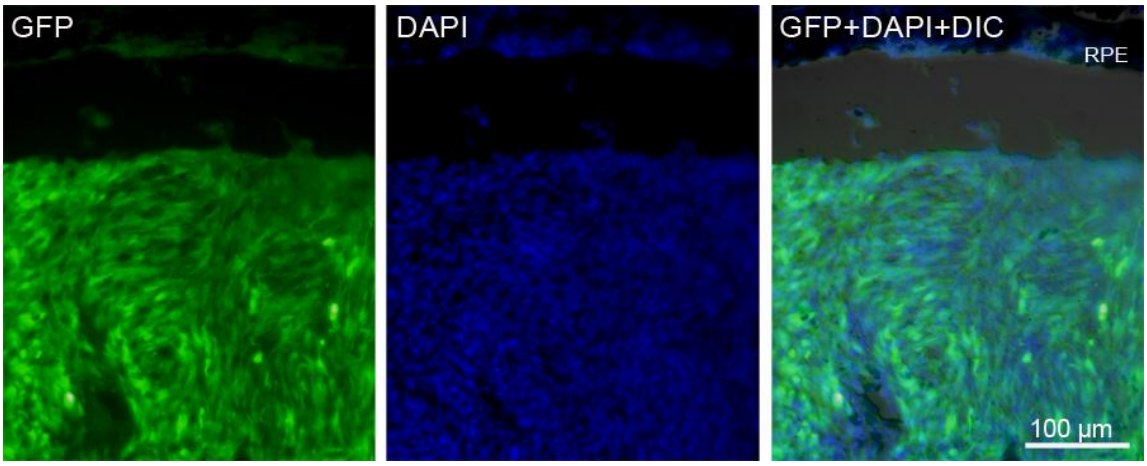


Figure 1. Survival of transplanted MSCs in the host retina of MYOC-mouse.

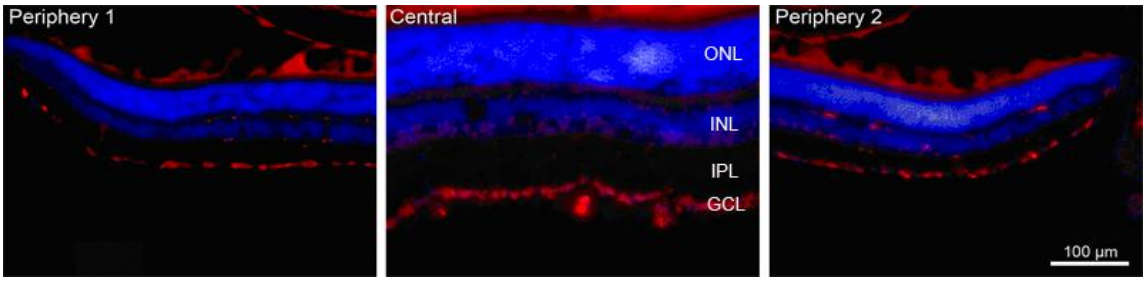
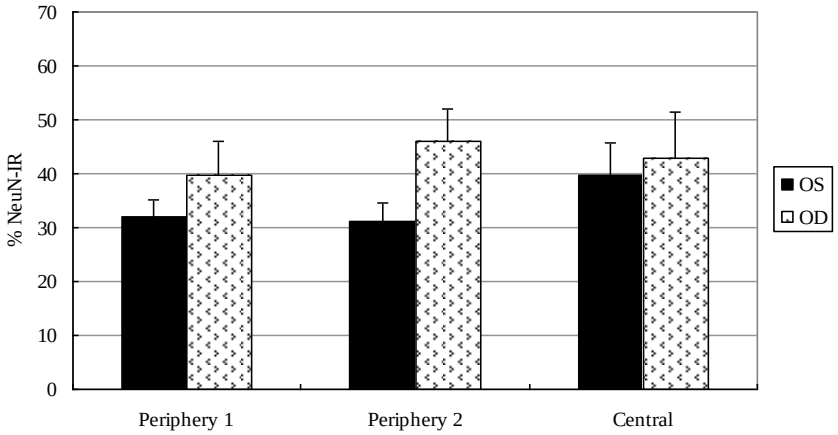


Figure 2. Representative images showing NeuN-IR cells in the GCL of the host retina.

(A) Transplant of BDNF-MSCs



(B) Transplant of GFP-MSCs

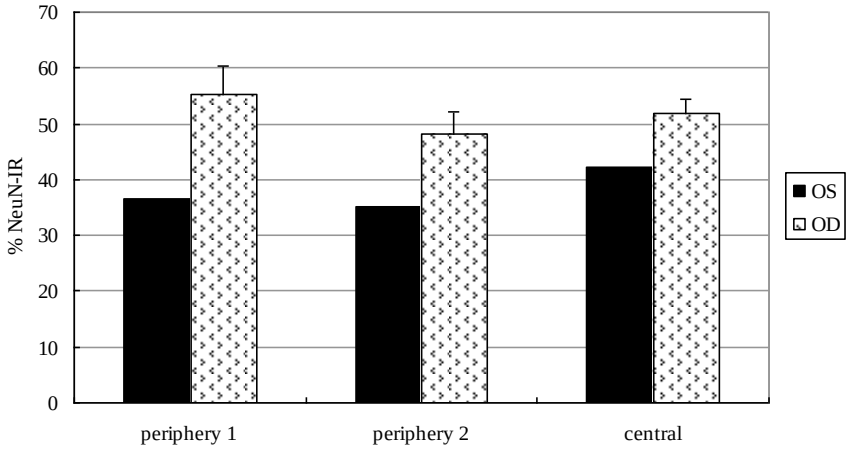


Figure 3. Survival of retinal ganglion cells (NeuN-IR) in the adult MYOC-mice retina.

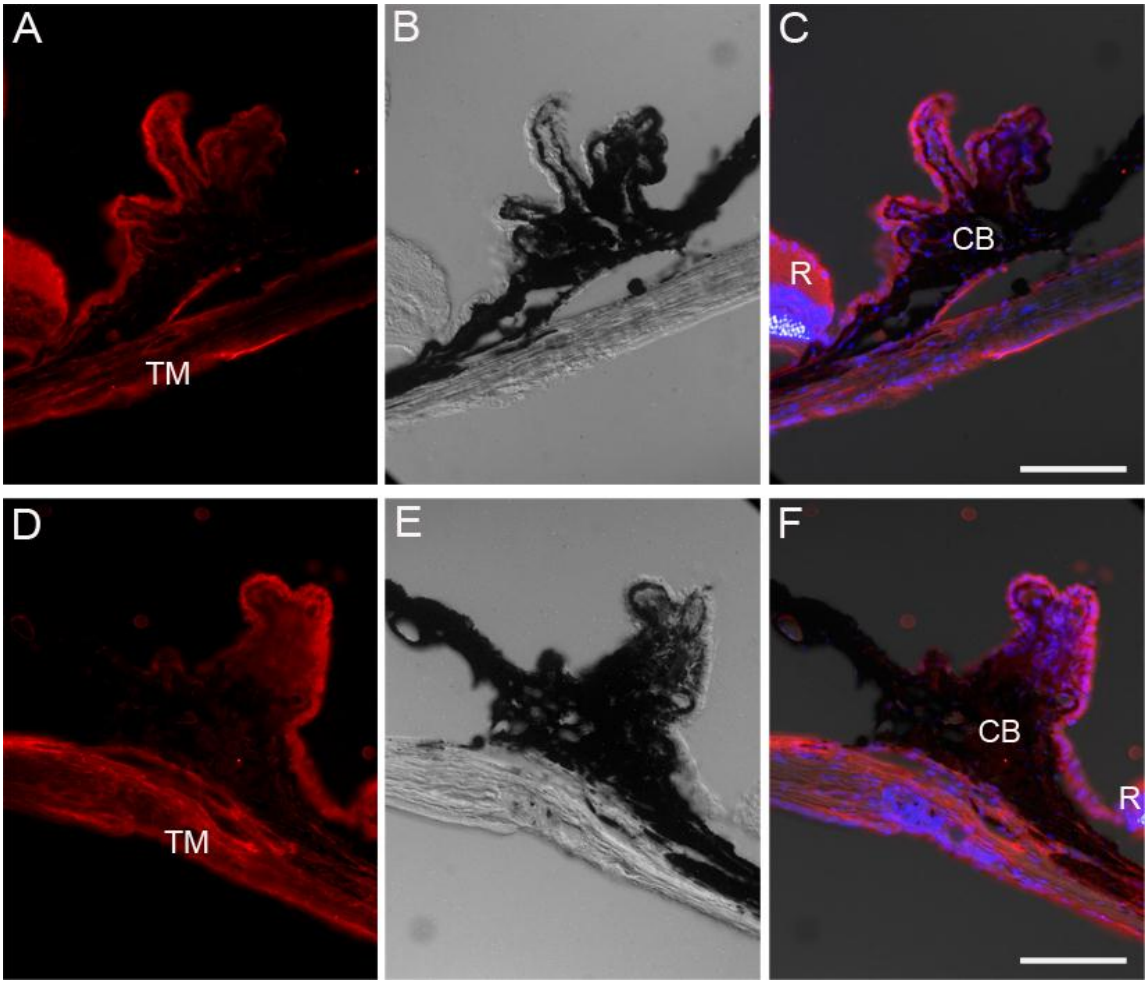


Figure 4. Upregulated expression of myocilin protein on the trabecular meshwork after MSC transplantation.

CHAPTER 6: GENERAL CONCLUSIONS

Firstly, the studies presented in this dissertation are the first to characterize bone marrow MSCs from Brown Norway rats and demonstrate their capability to promote neurite outgrowth from neuronal AHPCs and stimulate oligodendrocyte differentiation. Secondly, the studies describe, for the first time, the potential of genetically modified MSCs as a cellular vehicle of neurotrophic factors for the eye of transgenic mouse models of glaucoma. Our strategy of cell-based therapy consists of *in vitro* studies, engineering MSCs to make them secrete BDNF and/or GDNF, and the following *in vivo* studies, perform an intraocular transplantation into the eyes of two different models of transgenic mice (DBA/2J and Myocilin-mutant mice).

These studies provide a new resource of bone marrow MSCs and this broadens the opportunity of MSC utilization in a variety of research fields, including neurodegenerative diseases and injuries. Also, our *in vivo* studies provide a clue to the development of long-term therapeutic strategy for glaucomatous retinas.

We have made a number of important observations in these studies, including:

1. Mesenchymal stem cells (MSCs), derived from the bone marrow of Brown Norway rats, can be easily isolated and expanded in culture for a sustained period of time. MSCs were healthy and showed stable and consistent growth rate.
2. Rat MSCs were capable of stimulating neurite outgrowth from adult hippocampal progenitor cells (AHPCs). Also, MSCs promoted morphological differentiation of oligodendrocytic AHPCs, examined by co-culture.

3. We successfully engineered mouse MSCs and they secrete increased amount of bioactive Brain-derived neurotrophic factor (BDNF) and/or Glial cell-derived neurotrophic factor (GDNF).

4. We have demonstrated that engineered mouse MSCs could survive up to 4 months post transplant (PT) in the adult host retinas of DBA/2J mice.

5. We also have shown that engineered MSCs could survive up to 1 year post transplant in the adult host retinas of Myocilin-mutant mice.

6. We have found that a potential role of MSCs on the reduction of glial activation after MSC transplantation into the DBA/2J host retina.

7. We have found that potential neuroprotection, following MSC transplantation, on the retinal ganglion cells (RGCs) in the retina, RGC axons in the optic nerve, and retinal architecture on the inner retina.

The final conclusion of this dissertation is that bone marrow derived MSCs are a potent cell population capable of promoting neuronal and oligodendrocytic differentiation from brain progenitors. Furthermore, genetically modified MSCs, which secrete BDNF and/or GDNF, have potential to provide neuroprotection on degenerating host retinas and optic nerves.

RECOMMENDATIONS FOR FUTURE RESEARCH

The studies presented in this dissertation provide very important evidence showing the capacity of bone marrow-derived MSCs *in vitro* as well as *in vivo*.

Our *in vivo* studies using hereditary glaucoma models, that mimic human glaucoma, and MSCs, as a potential vehicle of neurotrophic factors, provide a good step to develop strategies of cell-based therapy for human glaucoma. Future studies to be performed in these mouse models would be detection of neurotrophic factors *in vivo* after MSC transplantation. Using very sensitive detection technique, such as High-performance liquid chromatography (HPLC) or western blots would allow us to detect even very small amount of BDNF or GDNF from the vitreous humor of the host eyes. Additionally, performing behavioral tests would provide more convincing evidence demonstrating successful neuroprotection. Possible behavioral tests that are available for rodents include 1) Morris water maze and swim task, which test central vision, and 2) Rotarod measurements, which test vision and vestibulomotor function, and 3) assessment of optokinetic response, which evaluate visual acuity and contrast sensitivity. Also, investigation of other potential neuroprotective proteins, such as NT3, NT4, NGF, and CNTF, and the combination of the factors would be very useful. In our in vivo studies, increasing sample size per each transplant group will be required to evaluate the significance of the effects following MSC transplantation.

Since physiological phenomena *in vivo* are very complex, we do not know which types of retinal cells respond and which signaling pathways are activated or inactivated in response to BDNF, GDNF, or the combination of both factors in the host retinas. Further experiments to

reveal changes in molecular aspects will be required for better understanding of neuroprotection.

Considering of clinically more relevant trials, the next step in the series of these studies would be to investigate the effectiveness of MSC transplant in large animal models, such as cat, dog, or pig. These animals would be more appropriate to study human glaucoma, than rodents. They have a larger size of eyes than rodents' and also they allow us to examine our strategy for a longer period of time, up to years. Outcome from our studies will be supportive to develop clinical therapies in the humans.